Evaluation of novel host markers detected in plasma and saliva as biosignatures for the rapid diagnosis of TB disease and monitoring of the response to TB treatment

by
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Declaration

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Abstract

BACKGROUND:

There is an urgent need for new tools for the rapid diagnosis of tuberculosis (TB) disease and monitoring of the response to treatment.

OBJECTIVES:

To investigate the usefulness of host markers detected in plasma and saliva, as well as antibodies against *M. tuberculosis (M.tb)* antigens, as biomarkers for the diagnosis of TB disease and monitoring of the response to treatment. To investigate the usefulness of a diagnostic approach involving the combination of antibodies and cytokines as a tool for diagnosing TB disease.

METHODS:

We prospectively collected plasma and saliva samples from individuals that presented with symptoms requiring investigation for TB disease at a health centre in Cape Town, South Africa, prior to the establishment of a clinical diagnosis. Patients were later classified as having TB disease or other respiratory diseases (ORD), using a combination of clinical, radiological and laboratory findings. The concentrations of host inflammatory biomarkers were investigated in plasma and saliva samples from all study participants using a multiplex platform, whereas antibody responses against seven *M.tb* antigens, were investigated by ELISA. The diagnostic accuracies of individual biomarkers were assessed by receiver operator characteristics (ROC) curve analysis, whereas the accuracies of combinations between different biomarkers were assessed by General Discriminant Analysis (GDA).

RESULTS:

Of the 74 host markers evaluated in plasma, 18 showed diagnostic potential as determined by area under the ROC curve (AUC), with the most promising being NCAM, CRP, SAP, IP-10, ferritin, TPA, I-309, and MIG, which diagnosed TB disease individually with AUC ≥0.80. A six-marker plasma protein biosignature comprising of NCAM, SAP, IL-1β, sCD40L, IL-13 and Apo A-1 diagnosed TB disease with a sensitivity of 100% (95% CI, 86.3-100%) and specificity of

89.3% (95% CI, 67.6-97.3%), irrespective of HIV status, whereas six-marker plasma protein biosignatures diagnosed TB disease with 100% accuracy in the absence of HIV. Of the 69 host markers that were investigated in saliva, only two (IL-16 and IL-23) showed diagnostic potential with AUC ≥0.70. A five-marker salivary biosignature comprising of IL-1β, IL-23, ECM-1, HCC1 and fibrinogen diagnosed TB disease with a sensitivity of 88.9% (95% CI,76.7-99.9%) and specificity of 89.7% (95% CI, 60.4-96.6%), regardless of HIV infection status, whereas eight-marker salivary biosignatures performed with a sensitivity of 100% (95% CI, 83.2-100%) and specificity of 95% (95% CI, 68.1-99.9%) in the absence of HIV infection. IgA responses against four *M.tb* antigens (NarL, Rv3019c, "Kit1" and "Kit2") were significantly different between TB patients and individuals with ORD, with combinations between different antibodies diagnosing TB disease with an AUC of 0.80. The diagnostic accuracy of the antibodies increased when used in combination with patient's symptoms or cytokines. Finally, the concentrations of biomarkers detected in plasma and saliva changed during TB treatment, thereby indicating that they may be useful in monitoring of the response to TB treatment.

CONCLUSIONS:

We have identified novel plasma and salivary biosignatures which may be useful in the diagnosis of TB disease and monitoring of the response to TB treatment. Our findings require further validation in larger studies.

Opsomming

AGTERGROND

Daar is 'n dringende behoefte aan nuwe toestelle vir die vinnige diagnose van tuberkulose (TB) en monitering van die reaksie op behandeling.

DOELWITTE

Om die nut van gasheer merkers in plasma en speeksel waar te neem , sowel as teenliggaampies teen *M. tuberculosis* (*M.tb*) antigene , as biomerkers vir die diagnose van TB en monitering van die reaksie op behandeling te ondersoek . Om die nut van 'n diagnostiese benadering met betrekking tot die kombinasie van teenliggaampies en sitokiene as 'n toestel vir die diagnose van TB te ondersoek.

METODES

Ons het plasma en speeksel monsters van individue met simptome wat tot die ondersoek van TB dui vooruitwerkend ingesamel by 'n gesondheidsentrum in Kaapstad , Suid-Afrika , voor die vestiging van 'n kliniese diagnose. Pasiënte was later geklassifiseer as TB of ander respiratoriese siektes (ARD) pasiënte, met behulp van 'n kombinasie van kliniese , radiologiese en laboratorium bevindings. Die konsentrasies van die gasheer inflammatoriese biomerkers in al die studie deelnemers in plasma en speeksel monsters was ondersoek met behulp van 'n multiplex platform , terwyl teenliggaam response teen sewe *M.tb* antigene , ondersoek was met ELISA . Die diagnostiese akkuraatheid van individuele biomerkers is beoordeel deur ontvanger operateur eienskappe (OOC) kurwe analise ,terwyl die akkuraatheid van kombinasies tussen verskillende biomerkers beoordeel was deur Algemene Diskriminant Analise (GDA) .

RESULTATE

Van die 74 gasheer merkers wat geëvalueer was in plasma het 18 diagnostiese potensiaal gehad soos bepaal deur area onder die OOC kurwe (AOC), met NCAM, CRP, SAP, IP-10, Ferritin, TPA, I-309, en MIG as die mees belowende merkers wat TB individueel diagnoseer

met AOC ≥0.80. 'n Ses-merker plasmaproteïen biosignature bestaande uit NCAM, SAP, IL-1β, sCD40L, IL-13 en Apo A-1 het TB gediagnoseer met 'n sensitiwiteit van 100% (95% CI, 86,3-100%) en spesifisiteit van 89,3 % (95% CI, 67,6-97,3%), ongeag MIV-status, terwyl ‘n ses-merker plasmaproteïen biosignatures TB gediagnoseer het met 100% akkuraatheid in die afwesigheid van MIV. Van die 69 gasheer merkers wat ondersoek was in speeksel het slegs twee (IL-16 en IL-23) diagnostiese potensiaal getoon met AOC ≥0.70. 'n Vyf-merker speeksel biosignature bestaande uit IL-1β, IL-23, ECM-1, HCC1 en fibrinogeen het TB gediagnoseer met 'n sensitiwiteit van 88,9% (95% CI, 76,7-99,9%) en spesifisiteit van 89,7% (95% GI, 60,4-96,6%), ongeag van MIV-infeksie status, terwyl agt-merker speeksel biosignatures gegenereer was met 'n sensitiwiteit van 100% (95% CI, 83,2-100%) en spesifisiteit van 95% (95% CI, 68,1-99,9 %) in die afwesigheid van MIV-infeksie. IgA reaksies teen vier *M.tb* antigene (NarL, Rv3019c, "Kit1" en "Kit2") het aansienlik verskil tussen TB-pasiënte en individue met ARD, met kombinasies tussen verskillende teenliggaampies wat TB diagnoseer met 'n AOC van 0.80. Die diagnostiese akkuraatheid van die teenliggaampies verhoog wanneer dit gebruik word in kombinasie met pasiënt simptome of sitokiene. Ten slotte, die konsentrasies van biomerkers wat in plasma en speeksel ondersoek was verander tydens TB behandeling, en dui sodoende aan dat hulle nuttig kan wees in die monitering van die reaksie op TB behandeling.

GEVOLGTREKKING

Ons het nuwe plasma en speeksel biosignatures geïdentifiseer wat nuttig kan wees in die diagnose van TB en monitering van die reaksie op TB behandeling. Ons bevindinge vereis verdere bekragtiging in groter studies .

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List of Abbreviations

°C	Degree Celsius
µl	Microliter
A2M	alpha-2-macroglobulin
ANOVA	analysis of variance
Apa	Alanine and proline rich secreted protein
Apo	apolipoprotein
AUC	Area Under Curve
BCG	Bacillus Calmette –Guerin
BCA-1	B-cell attracting chemokine
BDNF	Brain-Derived Neurotrophic Factor
CC3	Complement Component 3
CD	Cluster of Differentiation
CFH	Complement factor H
CFP-10	Culture filtrate protein-10
CRP	C-reactive protein
DOTS	Directly observed therapy short course
ECM1	Extracellular matrix protein 1
EDCTP	European & Developing Countries Clinical Trials Partnership
ELISA	Enzyme-Linked Immunosorbent assay
EMB	Ethambutol
ENA-78	Epithelial neutrophil activating protein
ESAT-6	Early secretory antigenic target-6
Esxr	ESAT-6 like protein
EQAPOL	External Quality Assurance Program
GCP2	Granulocyte chemotactic protein-2
GDA	General Discriminant Analysis
GDF -15	Growth differentiation factor

H_2SO4	Sulphuric acid
HCC1	Hemofiltrate CC chemokine-1
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGRA	Interferon Gamma Release Assay
IL	Interleukin
INH	Isoniazid
IP	Interferon gamma inducible protein
I-TAC	Interferon inducible T-cell alpha chemoattractant
IUATLD	International Union Against Tuberculosis and Lung Disease
LAM	Lipoarabinomannan
LTBI	Latent TB infection
LSD	Least Significant Difference
MDC	Minimum detectable concentration
MDR-TB	Multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Inhibitor Tube
MIG	Monokine induced by gamma interferon
MIP-1β	Macrophage inflammatory protein
MIP-4	Macrophage inflammatory protein-4
mm	millimetres
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MSD	Meso Scale Discovery
M.tb	*Mycobacterium tuberculosis*
NarL	Nitrate/nitrite response transcriptional regulatory protein
NCAM	Neural Cell Adhesion Molecule

nm	nanometres
NIH	National Institutes of Health
NPV	Negative predictive Value
ORD	Other respiratory diseases
PAI-1	total Plasminogen Activator Inhibitor-1
PCT	Procalcitonin
PEDF	Pigment epithelium derived factor
POC	Point-of-Care
PPV	Positive Predictive Value
PPD	Purified Protein Derivative
PstS1	Periplasmic phosphate-binding lipoprotein
PZA	Pyrazinamide
RD1	Region of Difference
RIF	Rifampicin
ROC	Receiver Operator Characteristics
SAA	Serum Amyloid A
SAP	Serum Amyloid P
SCF	Stem Cell Factor
SDF-1α	Stromal cell Derived Factor-1 alpha
TB	Tuberculosis
Th1	T helper 1
TNF	Tumor Necrosis Factor
Tregs	regulatory T-cells
TPA	Tissue Plasminogen Activator
TPO	Thrombopoietin
TST	Tuberculin Skin Test
TSLP	Thymic Stromal Lymphopoietin
VEGF	Vascular Endothelial Growth Factor
VDBP	Vitamin D Binding Protein

WHO	World Health Organisation
XDR-TB	Extremely drug resistant TB

Chapter 1

Introduction and Literature Review

1.1 Introduction

Tuberculosis (TB) is a leading death causing infectious disease, primarily infecting mammalian airways. TB is known to be caused by the *Mycobacterium tuberculosis (M.tb)* complex comprising of *M. tuberculosis, M. bovis, M. africanum, M. microtti and M. canetti,* with *M. tuberculosis* being the most prevalent in humans [1]. *M.tb* is an obligated aerobic bacillus, with a robust cell wall, with long- chain fatty acids and glycolipids [2]. It is categorised as a slow growing organism with a generation time of 18-48 hours, consequently causing TB disease progression to be reasonably slower compared to other infectious diseases [3]. The lungs are the most important site for the bacterium to manifest itself as the disease is transmitted almost exclusively by cough droplets from individuals with active pulmonary disease [4, 5]. These droplets (containing the bacilli) are subsequently inhaled into the respiratory tract where it infects the lungs that are highly aerobic and contains large measures of oxygen [6]. Though pulmonary TB occurs more frequently, extrapulmonary TB is also problematic as the bacilli can spread from the lungs to other parts of the body, through the lymphatic or blood circulating system, consequently causing extrapulmonary TB of the lymphatics, genitourinary system and meninges amongst others [7]. The global TB epidemic is also driven to a large extent by the human immunodeficiency virus (HIV) co-epidemic [8]. It is known that a higher risk of TB exists among HIV infected individuals [9]. Disseminated TB refers to two or more organs being infected simultaneously and it has been shown that HIV co-infection has further extended the possibility of mycobacterial dissemination due to immune deficiency, resulting in poor cell mediated immunity [10].

1.2 TB Epidemiology

Since the World Health Organisation (WHO) declared tuberculosis as a global emergency, TB mortality has fallen by 47% [9]. Despite the decline in the mortality percentage due to effective diagnosis and treatment of TB, disease burden remains remarkably significant. In 2014, six million new active tuberculosis cases and 1.5 million deaths (including 390,000 deaths among HIV-infected individuals) were reported worldwide [9]. The TB epidemic seems to be of even greater concern with the occurrence of multi-drug resistant strains of *M.tb*. It has been estimated that 3.3% of new TB cases and 20% of previous cases have multidrug-resistant tuberculosis (MDR-TB).It has been recognised that regions in Africa had the highest rates of TB per capita (281 cases per 100 000 people) [9].

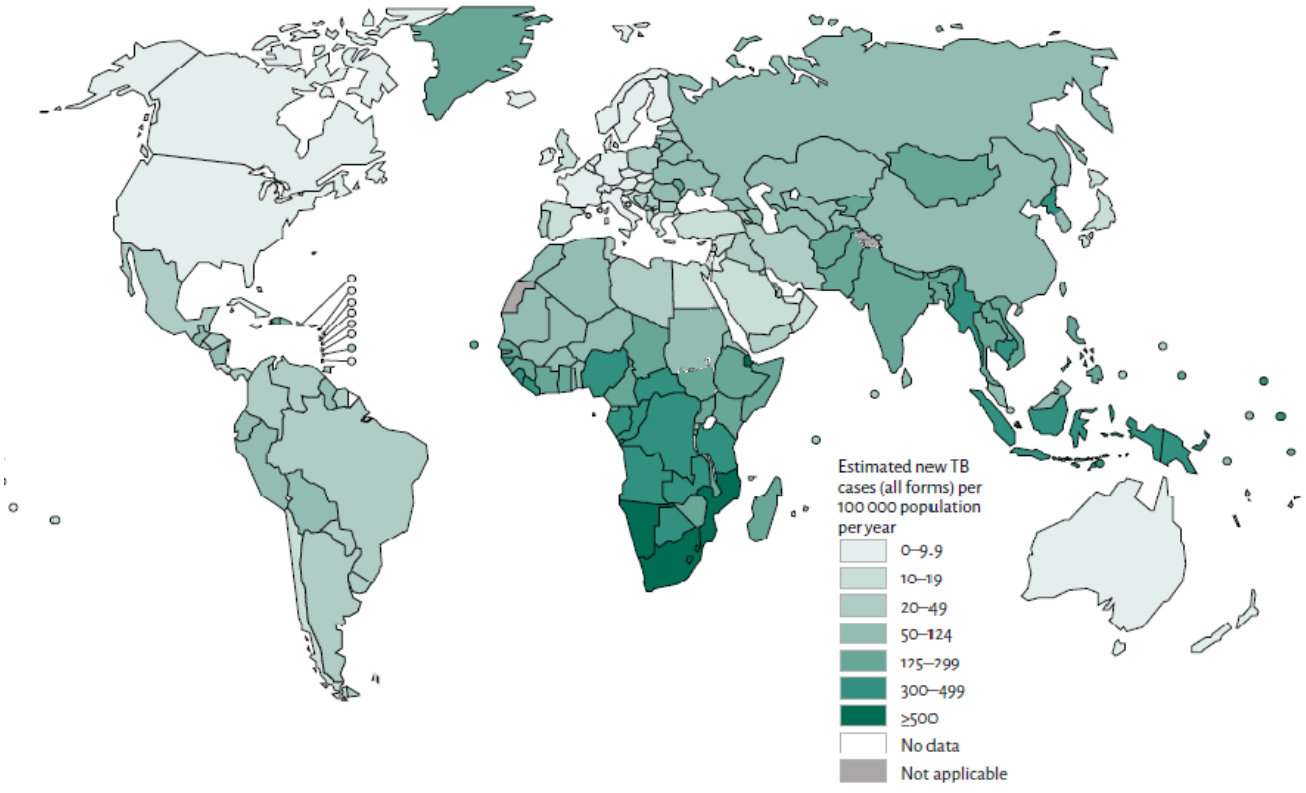

Figure 1.1: World map indicating the estimated TB incidence rate in 2014. Source: WHO TB report 2015 [9].

1.3 General Immunology

The human body is exposed to various microbial organisms on a daily basis. Fortunately, the body's immune system is distinctively developed to prevent infections and to eradicate established infections. This system of defence is mediated by a collection of immune cells and several other molecules. Immune cells are primarily known to develop in the bone marrow. However, the maturation and activation of these cells generally occurs in the secondary lymphoid organs involving the thymus, spleen and lymph nodes [11, 12]. These cells are able to migrate between tissues and interact with one another ultimately leading to a specific immune response. The human immune system has been divided into two categories; innate and adaptive immunity [13].

Innate immunity can be seen as the body's first line of defence that aids in protecting against common pathogens by means of a rapid and non-specific response. In contrast, adaptive immunity is more specific and therefore enables a more effective response [13]. These two types of immune responses respectively have their own specific cells that are responsible for their protective functions. Innate immunity consists of mast cells, basophils, eosinophils and phagocytes that are responsible for ingesting material that the body perceives as foreign [13]. Circulating phagocytes include neutrophils which are mostly found in areas of inflammation, monocytes, which differentiate into macrophages and dendritic cells that have essential antigen presentation functions [14–16]. Furthermore the innate immune system ,in part, initiates and activates the adaptive immune system [17]. B, as well as T-cells are key mediators of the adaptive immune system[13]. B-cells are known to produce antibodies that are responsible for eliminating extracellular microbial antigens. These antibodies also have the ability to mediate responses within the innate immune system by amplifying the ingestion of phagocytes [18, 19]. The two main T-cells are the cluster of differentiation (CD)4^+ cells, that aid in the production of antibodies and assist in phagocyte activation, and $CD8^+$ T-cells which are responsible for destroying virally infected cells [20, 21]. According to the cytokine secretion profiles, CD4 T cells can be classified as T helper 1 (Th1), Th2 or regulatory T-cells (Tregs) [20]. In addition unconventional T-cells such as gamma/delta T-cells also exist and are known to have distinctive T-cell receptors on their surface [13]. Gamma/delta T-cells are

functionally known to exert cytotoxic activity. Studies have also shown that these T-cells can serve as a bridge between innate and adaptive immune responses [22].Additionally, innate and adaptive immune cells are able to produce molecules, called cytokines and chemokines, which are of particular importance during the development of the immune response by mediating division and differentiation of stem cells and activating lymphocytes (T-cells) and phagocytes [23–26].

1.4 Immune Responses to *M.tb* Infection

After exposure to *M.tb,* it is infrequent that the individual would go on to develop symptomatic disease. Therefore, although a third of the world's population is infected with *M.tb*, only about 5-10% actually develop active disease as others would be known as being latently infected (asymptomatic disease). This differential outcome of infection was demonstrated in 1926 in Lubeck, Germany, when infants were unintentionally vaccinated with a live *M.tb* strain instead of the *M. bovis* Bacillus Calmette -Guerin (BCG) vaccine strain [27]. Some of the infants went on to develop active TB disease, while others remained unaffected. This unfortunate event therefore demonstrated that some individuals have a natural immunity to *M.tb*, thereby revealing that the host immune response in certain individuals is adequate to protect against *M.tb.* Still an incomplete understanding remains as to why some individuals are protected against *M.tb* while others go on to develop disease. Although the protective mechanisms against *M.tb* are not fully understood, it is known that it involves an extensive range of both innate and adaptive immune responses [28, 29]. Earlier studies showed that the initial host response against *M.tb* involves the influx of phagocytes primarily including alveolar macrophages, neutrophils and dendritic cells [30]. Once the bacterium has been inhaled into the airways, it is taken up by alveolar macrophages and neutrophils [30]. Activated macrophages further initiate an immune response by inducing inflammatory responses via the production of various pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-12 and IL-18 as well as anti-inflammatory IL-10 [31–34]. In addition, chemokines including CCL2, CXCL10 are also produced to facilitate the recruitment and migration of additional macrophages and dendritic cells to the site of infection [33, 34]. Upon recognition of the invading bacteria, these dendritic

cells are also responsible for internalizing *M.tb* as well as upregulating the expression of various cytokines before migrating and maturing in draining lymph nodes, under the influence of IL-12, where they are responsible to prime naïve T-cells via antigen presentation towards a Th1 phenotype [35, 36]. Inflammation in the lungs causes effector CD4+ Th1 cells to migrate back to the site of infection (lungs) in a chemokine dependent manner where these cells produce TNF-α and Interferon-gamma (IFN-γ), which is needed for macrophage activation and the control of TB disease [37–39]. Flynn et al. demonstrated the importance of IFN-γ in the control of TB disease, and showed that IFN-γ gene depleted mice are highly susceptible to *M.tb,* with defects in the activation of macrophages and unrestrained bacilli growth [40]. IFN-γ is therefore important in determining susceptibility to TB disease as well as in determining disease severity and treatment outcome [41, 42].

Once the Th1 cells migrate to the lungs (during pulmonary TB) , granulomas are formed which are seen as the hallmark of *M.tb* infection [43]. TNF-α, produced by macrophages and $CD4^+$ T-cells, plays an important role in granuloma formation [44]. Granulomas are normally associated with highly activated immune responses and comprise of a collection of immune cells including macrophages, $CD4^+$-, and $CD8^+$- T cells, as well as B-cells [45]. These immune cells controls the infection by interacting with each other, subsequently resulting in an effective immune response, through the production of cytokines, activation of macrophages and T-cell responses that ultimately leads to killing *M.tb* [46–48]. Granulomas also contains the bacilli and thus prevents the spread of infection [49]. Consequently the resulting pathology can also create additional problems for the human host. Granulomas are also able to form an area of necrosis when excessive inflammation occurs, which would liquefy therefore providing infectious bacilli for further transmission [50, 51]. Studies also report that *M.tb* is able to induce apoptosis of macrophages that are located within the granuloma [52]. In addition to the importance of cell-mediated immunity in TB, humoral immunity is also significant in the fight against *M.tb*. Although *M.tb* is an intracellular pathogen, it is known that it also has an extracellular phase where it can be found in the upper respiratory tract during early infection also during more progressive phases after granulomas are ruptured [53].Therefore apart from their role in the granuloma, B-cells also produce antibodies that aid in the regulation of the induction of T-cell immunity against intracellular pathogens.

Moreover Rodriguez et al. demonstrated that after immunization, IgA deficient mice were found to be more susceptible to BCG infection in comparison to wild type mice [54]. Furthermore the analysis of cytokine responses demonstrated that IFN-γ and TNF-α production is reduced, thereby suggesting the importance of IgA in protecting against mycobacterial infections [54].

1.5 Diagnosis of TB Disease

An important aspect in the global control of TB disease includes the improvement of case finding, since several TB cases (about 3 million) are undiagnosed [55]. Additionally, this would result in a reduction in transmission of *M.tb* [55]. The clinical symptoms of pulmonary TB is a reflection of the host response to the bacterium. These symptoms include chronic cough, fever, weight loss, night sweats and hemoptysis [56]. Further examination findings would include radiological abnormalities such as lung cavities or densities [55]. However confirming the diagnosis of TB disease remains difficult as these clinical features are not specific to TB and overlap with other diseases such as lung cancer and pneumonia, resulting in delays before a practitioner would consider diagnosing a patient with active TB. Therefore in addition to clinical suspicion, employment of other methods are essential in order to confirm active TB disease.

1.5.1 Current Diagnostic Methods

Sputum smear microscopy is still the most frequently used method to determine the presence of mycobacteria, and is recommend by WHO and International Union Against Tuberculosis and Lung Disease (IUATLD) as the most appropriate method in TB endemic countries [57]. This method is relatively easy and cost effective, making it suitable for use in low-income countries. A more rapid form of this method exists, with the use of fluorescent staining and computer automated microscope reading, which would limit human errors especially false negative results [58]. However the use of this modified version would not be suitable in

resource-poor settings, due to high costs. Although sputum microscopy is highly specific, it has a variable sensitivity (32-97%) as the detection limit of microscopy is 10^4 bacteria/ml [59]. Studies have also shown that this method is less sensitive in individuals who are co-infected with HIV, and are more likely to result in having a negative sputum smear [60]. Furthermore, smear microscopy is unable to differentiate between *M.tb* and other nontuberculous mycobacteria and therefore requires further isolation and confirmation of *M.tb* [59]. One way of achieving this is through mycobacterial culture. Culture is regarded as the 'gold standard' for the diagnosis of TB disease. It is highly specific and more sensitive than microscopy, as only 10 bacteria/ml is needed for the detection of *M.tb* [61]. However since *M.tb* is a slow growing organism, it can take up to 6-8 weeks to confirm diagnosis. A recent advance in the TB diagnostic field was the automated Nucleic acid amplification technique, GeneXpert (Cepheid Inc., Sunnyvale, USA). This technique is able to deliver results within 2 hours, and is also able to detect resistance to rifampicin, as a proxy for MDR-TB [62]. It is highly sensitive and specific, compared to culture, with its sensitivity varying from 74-100% and specificity from 95-100% [63]. The use of GeneXpert is expensive and has high infrastructural needs that is not always obtainable in resource-poor settings, consequently creating a major obstacle for its use in these settings. If obtainable, in South Africa for example, the GeneXpert test is mostly available in centralized facilities, meaning that specimens collected at the peripheral level health care centres still have to be shipped to these central laboratories for testing. The usefulness of these sputum based tests is also limited in the difficult-to-diagnose patient groups, such as those with paediatric and extrapulmonary TB. A urine lipoarabinomannan (LAM) point-of-care lateral flow assay (Alere Determine^{TM} TB LAM Ag, Alere Inc, Waltham, MA, USA) has recently been developed and has been proven to be accurate for use as a rapid rule-in test for TB in hospitalised individuals with advanced immunosuppression due to HIV infection [64].

1.5.2 Immunological Diagnosis of TB Disease

M.tb infection (latent infection) is typically demonstrated by the host's reactivity to *M.tb* antigens. The tuberculin skin test (TST), a test which involves the administration of purified protein derivative (PPD) via intradermal injection, is the oldest *M.tb* infection diagnostic test, and has been in use for more than a century [65]. After administration of PPD, the ensuing

delayed type hypersensitivity immune response results in a visible induration that is measured 48-72 hours after injection [66]. The induration is measured in millimetres (mm)[66]. Although the TST is the cheapest and most widely available test for diagnosing *M.tb* infection, the test has several limitations as several factors are known to bring about false negative results including age, nutrition or immunosuppression [67]. Additionally, false positive results are a concern as PPD contains over 200 antigens that are also found in nontuberculous mycobacteria (NTM) and in the *M. bovis* BCG vaccine. It is therefore not highly specific [68].

Advances in genomic research led to the discovery of the early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), which are contained within the region of difference. As this region is present in pathogenic *M.tb* but absent in the BCG vaccine strains and most NTM, the discovery of ESAT-6 and CFP-10 led to the development of the so called Interferon Gamma Release Assay (IGRAs), which are now regarded as the gold standard tests for *M.tb* infection in some settings [69]. These assays are based on the principle that individuals who have been exposed to *M.tb* harbour pre-activated T cells, which respond rapidly, with the production of cytokines after re-challenge with *M.tb* specific antigens. Reactivity to these antigens in IGRAs is assessed through the measurement of IFN-γ produced after overnight culture in an enzyme-linked immunosorbent assay (Quantiferon TB Gold assays) or by the enzyme-linked immunosorbent technique in the case of the T.SPOT test [69][65]. Although IGRAs have been shown to be very useful in diagnosing *M.tb* infection, particularly compared to the TST, the use of these tests are restricted in high TB endemic areas, as these tests do not differentiate between latent *M.tb* infection and active TB disease. This is therefore one of the main setbacks in these settings because of the high prevalence of latent infection. Currently, there are no commercially available tests that are able to differentiate between latent and active TB disease. Serological diagnosis of TB disease is another immunological approach. However, the WHO banned the use of all commercially available serological diagnostic tests in 2011 [70].

1.5.3 New Approaches towards the Immunological Diagnosis of TB Disease

In order to improve the current situation where TB case detection and diagnostic capacity is suboptimum [9], it is important that rapid point-of-care (POC) tests that would be appropriate for use at community health care centres and which are cost-effective and sensitive, are developed. These POC tests should preferably be based on alternative sample types such as serum, saliva or urine, as tests based on sputum, including the existing microbiological tests, are not very suitable for use by individuals finding it difficult to provide decent quality sputum such as those with paediatric and extrapulmonary TB disease.

Much work is currently being done in the search for new, immunodiagnostic approaches for active TB disease. Amongst the approaches currently being investigated are T cell based approaches, involving the isolation of peripheral blood mononuclear cells and staining for the expression of surface biomarkers by flow cytometry after overnight stimulation with *M.tb* antigens [71, 72], investigation of new *M.tb* infection phase dependent antigens [73], other than ESAT-6 and CFP-10 [74, 75], investigation of new biomarkers (other than IFN-γ) produced after overnight stimulation of blood cells with new *M.tb* antigens [76],RNA biosignatures [77], serodiagnostic assays evaluating antibodies against new *M.tb* antigens [78, 79] amongst others. A prototype of a POC test for the measurement of host biomarkers detected in biological fluids has already been developed and the utility of this prototype investigated in several African countries [80].

Despite the potential shown by these previous studies, diagnostic tests are likely to make the most impact on the control of TB disease if based on *ex vivo* host markers detected in unstimulated samples, such as would be obtained in easily accessible samples including serum or saliva. Assays involving the use of these sample types would be more easily applicable for use at the POC especially in resource-limited settings. In previous antigen-stimulation studies by Chegou and colleagues, it was observed that some host markers performed best either individually or in multi-marker models when measured in unstimulated supernatants [81]. These included interferon (IFN)-α2, Interferon inducible protein (IP)-10, tumour necrosis

factor (TNF)-α, epidermal growth factor (EGF), transforming growth factor (TGF)-α and vascular endothelial growth factor (VEGF) [81]. A recent large multi-centred study involving the detection of *ex vivo* host inflammatory biomarkers in serum samples, showed that a combination of seven host markers (C-reactive protein, transthyretin, IFN-γ, complement factor H, apolipoprotein-A1, inducible protein 10 and serum amyloid A) could diagnose TB disease with 93.8% sensitivity and 73.3% specificity[82]. Furthermore, studies done on saliva from individuals with symptoms suggestive of pulmonary TB disease also demonstrated that salivary host inflammatory biomarkers have potential as diagnostic candidates for TB disease [83–85]. Given the promise shown by these previous studies, there is hope that *ex vivo* host inflammatory biomarkers, including markers detectable in serum, plasma or saliva could be beneficial in the diagnosis of TB disease at the POC, therefore highlighting the need for further investigations into such diagnostic biosignatures.

1.6 TB Treatment and Vaccines

Drug susceptible TB requires a combination of multiple antibiotics, known as the 'first line TB treatment regime'. This approach recommends the administration of four first-line drugs during the intensive phase of treatment namely isoniazid (INH), ethambutol (EMB), rifampicin (RIF) and pyrazinamide (PZA) , after which the continuation phase follows, for a minimum total treatment duration of 6 months [86]. All four drugs are administered for the first 2 months of treatment (in the intensive phase), followed by a further 4 months of treatment (continuation phase) with only INH and RIF. Directly observed therapy short course (DOTS) was implemented by WHO as a strategy to improve adherence due to the lengthy treatment of TB, and involves the directly observed administration of these drugs [87]. Despite the implementation of DOTS, MDR-TB still emerged, primarily due to non-compliance. MDR-TB is defined as resistance to INH and RIF. On the other hand, extremely drug resistant TB (XDR-TB) is defined by resistance to second-line drugs (used to treat MDR TB), consisting of fluoroquinolones and aminoglycosides [88]. Treatment for the resistant forms of TB remains difficult and requires a longer course of treatment. Furthermore treatment success remains low for drug-resistant TB, with high rates of treatment failure and mortality reported worldwide, with even earlier mortality reported in individuals co-infected with HIV [9]. In

order to successfully eradicate TB, the development of an effective vaccine is needed. In countries with high burdens of TB or HIV, BCG vaccination was integrated into childhood immunization programs, where BCG is intradermally administered at, or shortly after birth [89]. BCG vaccination has been shown to provide some protection against more dangerous childhood forms of TB, however protection decreases by adolescences, for reasons that are not seemingly clear [90, 91]. Furthermore the efficacy of BCG seems to vary between population groups and it has been proposed that host genetic variability and socioeconomic factors might be contributing towards this differential efficacy [92]. The variability of BCG vaccination has led to the development of various new vaccines that are currently in clinical trials.

1.6.1 Monitoring of the Response to TB Treatment

Monitoring of the response to TB treatment remains a challenge, due to the lack of appropriate tools. In most settings, monitoring of TB treatment response entails repeating the sputum smear microscopy test at month 2 after treatment initiation [93]. Conversion from smear-positive to smear- negative after 2 months of treatment is currently the only accepted biomarker for TB treatment response [93]. The many well-established limitations of sputum smear microscopy are therefore also limitations of this treatment efficacy assessment strategy, besides the fact that sputum smear microscopy cannot discriminate between dead and live mycobacteria. Chest x-rays are also used to assess TB treatment response. However, this is not possible in resource-poor settings, and it is also difficult to standardize the assessment of x-rays [94]. The use of culture to monitor TB treatment response has yielded conflicting results and the availability and long turnaround time of culture also limits its use for this purpose [95, 96]. Additionally, the GeneXpert test is also not suitable to monitor the efficacy of treatment, as it is unable to distinguish between DNA from live and dead bacteria [97]. Therefore, tests for monitoring TB treatment response are urgently needed worldwide, both for individual patient benefits and for assessment of the efficacy of new drugs. Host immunological biomarkers have been shown to have potential as tools for monitoring of TB treatment response, including the prediction of month 2 smear and sputum status [98]. However, no immunological tests currently exist for this purpose. In addition to the possibility

of easier incorporation into POC devices, host inflammatory biomarkers will be advantageous particularly if such tests are based on easily obtainable samples including serum, plasma or saliva. If measured early after the initiation of TB treatment, such biomarker based tests shall help in stratification of patients for standard or more intensive treatment. Therefore, there is a need for more work to be done on the identification of new biomarkers as well as validation of currently known potential candidates. Therefore in addition to the investigation of biomarkers as candidates for the diagnosis of TB disease, the utility of biomarkers as potential candidates for monitoring of TB treatment response was also assessed in the current thesis. Potential candidates could then be investigated further, in larger future cohort studies.

1.7 Study Objectives

1) To validate previously identified host biomarkers and to identify novel host biomarkers in plasma and saliva as diagnostic biosignatures for active TB disease

2.) To investigate the potentials of host biomarkers detected in plasma and saliva as candidates for monitoring of the response to TB treatment

3) To investigate a usefulness of antibodies against recently identified *M.tb* antigens as biomarkers for the diagnosis of active TB disease and monitoring of the response to TB treatment

4) To investigate a diagnostic approach in which multiple classes of antibodies against *M.tb* antigens are combined with host inflammatory biomarkers (cytokines, chemokines and acute phase proteins amongst others) as a combined tool for the diagnosis of TB disease

Chapter 2

Materials and Methods

2.1 Study Participants and Setting

The individuals who provided samples for the studies presented in this thesis were recruited through a large European & Developing Countries Clinical Trials Partnership (EDCTP)-funded biomarker study (the African European Tuberculosis Consortium; www.ae-tbc.eu). This project was conducted at various field sites serving seven institutions situated in six other African countries, with five European partner institutions. These field sites were study sites for the Amauer Hansen Research Institute, Ethiopia, Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia, Makerere University, Uganda, Karonga Prevention study, Malawi, MRC, The Gambia, The University of Namibia, Namibia and Stellenbosch University, South Africa. The study participants enrolled for the investigations presented in this thesis were recruited from the Fisantekraal Community Clinic in the outskirts of Cape Town, South Africa.

All the study participants presented with signs and symptoms requiring investigation for pulmonary TB disease, and were recruited prior to any clinical or laboratory assessments. All study participants were recruited between November 2010 and November 2012.

2.1.1 Inclusion Criteria

Participants were enrolled if they presented with persistent cough lasting ≥2 weeks and at least one of the following: fever, malaise, recent weight loss, night sweats, knowledge of close contact with a TB patient, haemoptysis, chest pain or loss of appetite. Participants were eligible for the study if they were 18 years or older and willing to give written informed consent for participation in the study, including consent for HIV testing.

2.1.2 Exclusion Criteria

Patients were excluded if they were pregnant, had not been residing in the study community for more than 3 months, were severely anaemic (haemoglobin <10 g/l), were on anti-TB treatment, had received anti-TB treatment in the previous 90 days or if they were on quinolone or aminoglycoside antibiotics during the past 60 days.

2.1.3 Ethics Statement

The study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (Reference no. N10/08/274).

2.2 Sample Collection and Preparation

In addition to providing samples that were used for routine diagnostic purposes, all the participants included in this thesis provided both plasma and saliva samples. Other samples including whole blood for serum separation, stimulation with different *M.tb* antigens and culturing, Quantiferon supernatants, urine, paxgene, DNA, peripheral blood mononuclear cells amongst others, were also collected from all study participants as required for the main study, for future studies.

2.2.1 Plasma Sample Collection and Preparation

Blood was collected into 6ml heparinized BD vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) and transported to the laboratory at 4-8^{O}C for further processing. Upon receipt in the laboratory, tubes were centrifuged at 2000 rpm for 10 minutes after which plasma was harvested, aliquoted and stored at -80 ^{O}C until used. Sample collection was repeated at months 2 and 6 after the start of TB treatment, only in individuals in whom TB disease was confirmed after diagnostic work-up.

2.2.2 Saliva Sample Collection and Preparation

Study participants fasted for at least one hour before saliva collection. Briefly, participants were asked to chew a sterile cotton swab (salivette) that was provided by the saliva collection kit manufacturer (Sarstedt, Numbrecht, Germany), for about 45 seconds. The swab was then removed from the participant's mouth with sterile forceps, inserted into a sterile tube provided by the manufacturer, and then transported to the laboratory at 4 - 8^{o}C. Upon arrival in the laboratory, the saliva samples were centrifuged at 1000g for 2 minutes and the supernatant harvested and stored at -80 ^{o}C until tested. After microbiological confirmation of TB disease in study participants, sample collection was repeated for the culture confirmed TB patients at month 2 and month 6 after the initiation of TB treatment.

2.3 Reference Standard for Diagnosing TB Disease

Routine diagnostic tests including mycobacterial cultures, sputum smears and chest radiography were performed on all study participants. Sputum samples were collected from all study participants and cultured using the Mycobacteria Growth Inhibitor Tube (MGIT) method (BD Biosciences, Franklin Lakes, NJ, USA). Positive MGIT cultures were examined for acid fast bacilli using the Ziehl-Neelsen technique (to check for contamination), followed by Capilia TB testing (TAUNS, Numazu, Japan), to confirm the isolation of organisms of the *M.tb* complex, before being designated as positive cultures. Sputum samples were also used to perform Ziehl- Neelsen sputum smear tests.

By using a combination of clinical, radiological, and laboratory findings, participants were classified as definite TB patients, probable TB patients, questionable TB patients and participants with other respiratory diseases (ORD) as described in detail in table 2.1.

Table 2.1: Case definitions used in classifying study participants (Reproduced from Chegou NN, et al. *Thorax* 2016)

Classification	Definition
Definite TB	Sputum culture positive for *M.tb* OR 2 positive smears and symptoms responding to TB treatment OR 1 Positive smear plus CXR suggestive of PTB
Probable TB	1 positive smear and symptoms responding to TB treatment OR CXR evidence and symptoms responding to TB treatment
Questionable	Positive smear(s), but no other supporting evidence OR CXR suggestive of PTB, but no other supporting evidence. OR Treatment initiated by healthcare providers on clinical suspicion only. No other supporting evidence
Other Respiratory Disease	Negative cultures, negative smears, negative CXR and treatment never initiated by healthcare providers

CXR, chest X-ray; *M.tb*, Mycobacterium tuberculosis; PTB, pulmonary TB.

2.4 Luminex Multiplex Immunoassay

The Bead-Based Luminex Multiplex Assay was used to determine the levels of 74 analytes in *ex-vivo* plasma and saliva samples from study participants. Briefly the Luminex Multiplex Immunoassay permits the quantitative simultaneous detection of a large array of soluble factors in a single sample (up to 500 biomarkers depending on the Luminex reader).

This assay is based on bead sets fixed with different intensities of dyes and pre-coated with analyte-specific antibodies. These beads are examined by two lasers (as is the case with the Luminex 200 instruments), which identifies the spectral property of the beads and therefore the levels of the associated analyte. The 74 different analytes investigated in the current thesis were selected based on their potentials as TB diagnostic or treatment response biomarkers, as identified in previous studies. Additionally markers that have not previously been investigated in the TB field, but which have been studied in other diseases (for example in lung cancer) were also included as we thought it would be interesting to investigate their

potentials in both plasma and saliva samples in the context of TB disease. The experiments were performed using kits supplied by Merck Millipore, Billerica, MA, USA and Bio- Rad Laboratories, Hercules, CA, USA as indicated in table 2.2 below. Experiments were conducted according to the instructions of the different manufacturers (Merck Millipore or Bio-Rad), in 96-well plates. However assay reagents for Luminex experiments were diluted 1:2 following the optimization experiments discussed in 2.8.1.

Briefly, after the preparation of all the reagents, controls and standards (according to the manufacturer's instructions) the standards, controls and samples were added to the appropriate wells, after which analyte-specific antibodies which were pre-coated onto color-coded magnetic microspheres (beads), was added to each well. After 2 hour (at room temperature) or overnight (at 4^{o}C) incubation on a shaker, depending on the type of kit, plates were washed using an automated magnetic bead washer (Bio-Rad). After addition of biotinylated detection antibodies, plates were incubated for 1 hour (for Milliplex kits or 30 minutes for Bio-Rad kits) followed by a further wash step (kits from Bio Rad) and addition of streptavidin-pycoerythrin (streptavidin-PE). After 30 minutes incubation (10 minutes for kits from Bio-Rad), plates were washed, followed by resuspension of the beads on a shaker. Plates were read using either the Bio-Plex 200 system or Bio-Plex Magpix. All incubation steps were done with agitation on a shaker at room temperature (or 4^{o}C) according to the speed recommended by the manufacturer. Beads were acquired and analysed using the Bio-Plex manager software, version 6.1 (Bio-Plex 200) or acquired using the Bio-Plex MP software, followed by analysis using the Bio-Plex Mananger 6.1, if the Magpix was used.

Table 2.2: Kits and analytes used for Luminex Multiplex Immunoassay. Please see the list of abbreviations for the full names of the different host markers. Host markers are also defined after first use in chapters 3 and 4.

Merck Millipore, Billerica, MA, USA	
Kits	**Analytes**
Human Cytokine/Chemokine Magnetic Bead Panel II (HCYP2MAG-62K-09)	BCA-1/CXCL13,ENA-78/CXCL5,I-309/CCL1,SCF,TSLP,TPO,SDF-1/CXCL12,IL-16,IL-28A
Human CD8+ T-Cell Magnetic Bead Panel (HCD8MAG-15K-06)	Granzyme B, sFas, sFasL, Granzyme A, Perforin,CD-137
Human Neurodegenerative Disease Magnetic Bead Panel 1 (HNDG1MAG-36K-05)	APOA1, APOC3, Complement C3, Complement Factor H, Prealbumin/ Transthyretin (TTR)
Human Neurodegenerative Disease Magnetic Bead Panel 3 (HNDG3MAG-36K-05)	BDNF, Cathepsin D, Myeloperoxidase (MPO), sNCAM/CD56, PAI-1 (total)
Human Cytokine/Chemokine Magnetic Bead Panel (HCYTOMAG-60K-11)	CD40L , IFN-G, IL-1B, TNF-A ,VEGF, IFN-A2, IL-12P40, IL-13, IP-10, MIP1B, TNFB
Human TH17 Magnetic Bead Panel (HTH17MAG-14K-08)	IL-17, IL-25, IL-17F, IL-21, IL-22, IL-23, IL-31, IL-33
Human Neurodegenerative Disease Magnetic Bead Panel 2 (HNDG2MAG-36K-04)-	α-2-Antitrypsin (A1AT), Complement C4, MIP4,PEDF
Human Cytokine/Chemokine Magnetic Bead Panel III (HCYP3MAG-63K-06)	LIX/CXCL6/GCP-2, IL-11, IL29, ITAC, MIG
Human Cytokine/Chemokine Magnetic Bead Panel	HCC1

III (HCYP3MAG-63K-01)	
Human Circulating Cancer Biomarker Magnetic Bead Panel 2 (HCCBP2MAG-58K-04)	Antithrombin III, Extracellular Matrix Protein 1 (ECM1), Vitamin D Binding Protein, Vitronectin
Human Cardiovascular Disease (CVD) Magnetic Bead Panel 2 (HCVD2MAG-67K-05)-	ADAMTS13,GDF15,Myoglobin,NGAL/Lipocalin-2,sP-Selectin
Human MMP Magnetic Bead Panel 2 (HMMP2MAG-55K-02)	MMP-2, MMP-9
Bio- Rad Laboratories, Hercules, CA, USA)	
Human Acute Phase Multiplex 4 Plex Panel	A2M, Haptoglobin, CRP, SAP
Human Acute Phase Multiplex 5 Plex Panel	PCT, Ferritin, TPA, Fibrinogen, SAA

Prior to analysis, samples for HMMP2MAG-55K-02 were diluted 1:2, samples for HNDG3MAG-36K-05, HCYP3MAG-63K-01, HCCBP2MAG-58K-04, HCVD2MAG-67K-05 and Human Acute Phase Multiplex 5 Plex Panel were diluted 1:100 , samples for HNDG2MAG-36K-04 were diluted 1:2000, while samples for HCCBP2MAG-58K-04 were diluted 1:10 000 and HNDG1MAG-36K-05 diluted 1:40 000, whereas those for HCYTOMAG-60K-11,HCYP2MAG-62K-09,HCD8MAG-15K-06, HTH17MAG-14K-08, HCYP3MAG-63K-06 were evaluated neat (undiluted) following previous optimization experiments or recommendations from the manufacturer.

2.5 Meso Scale Discovery (MSD) Assay

The Meso Scale Discovery (MSD) platform is a relatively new platform that is being marketed as an alternative to the Luminex platform, for biomarker discovery purposes. Our laboratory recently acquired a MSD SQ120 instrument and as done for the optimization experiments

reported in 2.8.1, it was necessary for us to demonstrate that the equipment was fit-for-purpose before introducing it for routine biomarker discovery and/or validation work in the laboratory. We therefore designed an experiment in which we compared the results obtained from the MSD platform to the well-established Luminex platforms (section 2.8.2).

To investigate whether results generated by the two multiplex platforms currently used in our laboratory (Luminex and MSD) were comparable, plasma samples were similarly thawed, aliquoted and then analysed separately (same plasma samples on the different platforms). The Luminex kits containing the seven common analytes (CRP, SAA, IFN-γ, IL-1β, IL-13, TNF-α and MMP-9) were purchased from Merck Millipore and Bio-Rad as follows: CRP and SAA assays were purchased from Bio-Rad, whereas customized kits containing IFN-γ, IL-1β, IL-13, TNF-α and MMP-9 were purchased from Merck Millipore. All assays were performed according to the different manufacturers' instructions (as described in section 2.4 for the Luminex platform). Experiment results are discussed in section 2.8.2.

Briefly, MSD platform enables the detection and quantitation of biomarkers and signalling molecules in simple and complex matrices, by electrochemiluminescence detection, which uses labels that emit light (SULFO-TAG labels) when electrochemically stimulated. Each well in a MSD plate contains electrodes that are coated with capture antibodies, allowing the analyte to be captured on the electrode. Upon electrochemical stimulation, light emits at the surface of the electrode, therefore allowing the concentration of the analyte to be determined. Background signals are believed to be minimal with this technology as the stimulation mechanism (electricity) is decoupled from the signal (light). Multiple cycles of each label amplify the signal to enhance light levels and improve sensitivity. The instruments use custom-designed optics and ultra-sensitive photo detectors to detect and quantitatively measure light emitted from the microplates after which electronic and signal processing algorithms convert the measured signal to useful data in real time (Figure 2.1).

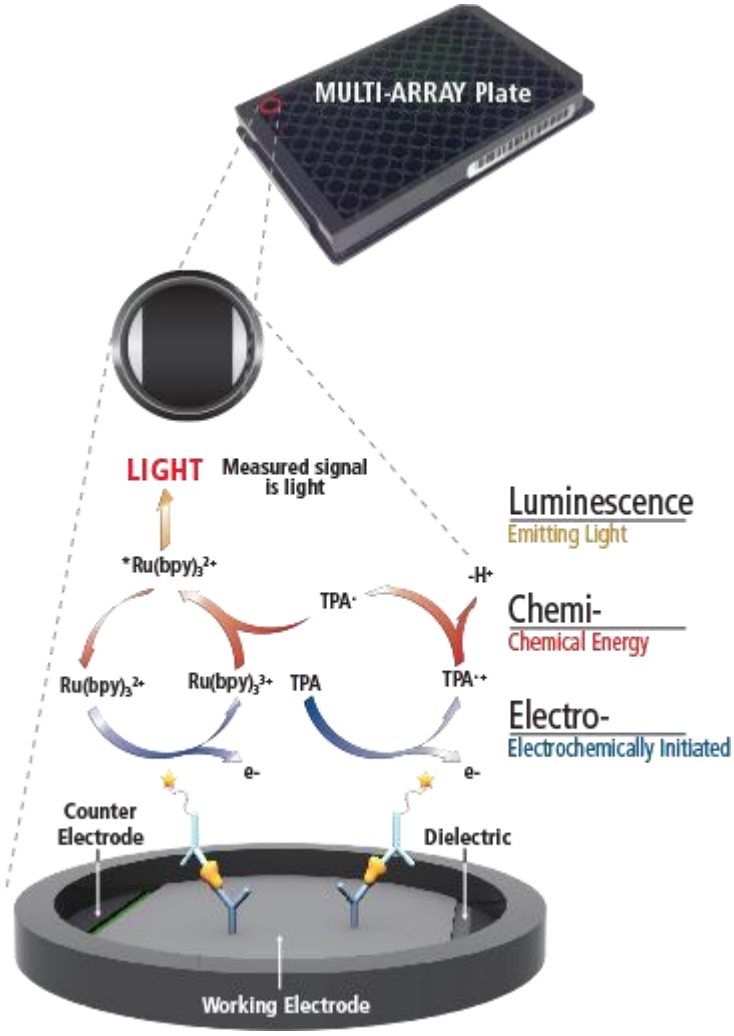

Figure 2.1: Principle of the Meso Scale Discovery technology as published by the company on https://www.mesoscale.com/en/technical_resources/our_technology/ecl. Biological reagents are attached to high binding carbon electrodes in the bottom of the MSD plates. The electrochemiluminescent labels employed in the assay (SULFO-TAG) are conjugated to detection antibodies, and allow for ultra-sensitive detection of analytes. After the MSD instrument applies electricity to the plate electrodes, the SULFO-TAG labels emit light, which is captured by an in-built camera. The intensity of the light emitted is proportional to the amount of analyte in the sample. Image downloaded from: https://www.mesoscale.com/en/technical_resources/our_technology/ecl

MSD assays were performed as follows: after the preparation of reagents (according to the manufacturer's instructions), samples, standards and controls were added to the appropriate wells in their respective 96-well plates and incubated for 2 hours at room temperature, with

agitation according to the speed recommended by the manufacturer. The plates were then washed 3 times with wash buffer after which detection antibody solution was added and incubated at room temperature with shaking for 2 hours. After incubation, the wash step was repeated after which 2X read buffer was added to each well and the plate read on the MSD QuickPlex SQ120 instrument.

2.6 Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent assay (ELISA) was used for the detection and quantitative determination of immunoglobulin (Ig) A, Ig G and Ig M antibodies against seven *M.tb* specific antigens (Table 2.3). Plasma samples were thawed and pre-diluted (1:5) with 87% glycerol and stored at -80 ^{O}C until needed for the ELISAs. On the day of the ELISA, the pre-diluted samples were further diluted (1:200) with sample diluent, following the optimization experiments described below (section 2.8.3). The experiments were performed according to manufacturer's instructions (LIONEX Diagnostics and Therapeutics, Braunschweig, Germany). Briefly, after the preparation of reagents (according to the instructions of the manufacturer), diluted samples and standards were added to appropriate wells in 96-well plates containing immobilized purified recombinant antigens that were bound to the surface of the plates. After 1 hour incubation at 37^{o}C the plates were washed 3 times with diluted wash buffer. This was followed by the addition of the ready- to- use conjugate to each well and a further incubation for 30minutes at 37^{o}C. After repeating the wash step, ready-to –use substrate solution was added to all wells, followed by incubation for 20 minutes at 37^{o}C in the dark. The substrate reaction was terminated by the rapid addition of ready-to-use stop solution (5% H_2SO4) into each well. The plate was then read on an ELISA plate reader (iMark™ Microplate absorbance reader, Bio-Rad, USA) by measuring the optical density at 450 nanometres (nm) and reference wavelength of 650nm.

Table 2.3: Recombinant antigens of *M.tuberculosis* used in the study

M.tuberculosis antigens	Mol mass (kDa)	Rv number	Ig Class
EsxR (TB10.3)	36	Rv3019c	IgA
PstS1	37.37	Rv0934	IgA
"Kit 1"	*	*	IgA
"Kit 2"	*	*	IgA
Apa	32.7	Rv1860	IgA
NarL	23.9	Rv0844c	IgA
LAM	-	-	IgM

*Name of kits are not revealed due to intellectual property concerns with the manufacturer. Periplasmic phosphate-binding lipoprotein PstS1; Alanine and proline rich secreted protein Apa; Nitrate/nitrite response transcriptional regulatory protein NarL; lipoarabinomannan LAM , kDa kilo Dalton

2.7 Statistical Analysis

All statistical analyses were conducted using Statistica (Statsoft, Ohio, USA) and Graphpad Prism version 5 (Graphpad Software Inc., CA, USA). Differences between any two groups compared were analysed using the Mann-Whitney U test, if the data was not normally distributed, whereas the student's t-test was used if the data was normally distributed. The diagnostic abilities of individual host markers and antibody responses for TB disease were assessed by receiver operator characteristics (ROC) curve analysis. Cut-off values that resulted in the highest combination of sensitivity and specificity were selected. The predictive abilities of combinations of host markers and/or antibodies were investigated by general discriminant analysis (GDA), with leave-one-out cross validation. Differences in the levels of host markers and antibodies during the course of TB treatment were analysed using mixed model repeated measures analysis of variance (ANOVA), with Fisher's Least Significant Difference (LSD) post hoc testing. P-values ≤0.05 were considered significant. Statistical analyses were conducted with the assistance of a statistician from the Department of Statistics and Actuarial Sciences, Centre for Statistical Consultation, Stellenbosch University.

2.8 Results of Optimization Experiments

2.8.1 Optimization of Beads, Biotinylated Detection Antibodies and Streptavidin-phycoerithrin for use in Luminex Diluted Assays

Dilution of reagents prior to usage in Luminex experiments is commonly performed in research laboratories worldwide, due to costs. As these protocols were introduced to our laboratory by our collaborators, there was a need to prove that assay reagents diluted as such still yielded the desired results, prior to implementation of the protocols in the laboratory. Therefore prior to performing the Luminex experiments described in the current thesis, the assay was optimized for the use of beads, streptavidin-PE and biotinylated detection antibodies with different dilutions that have been proposed by users in other laboratories. This reagent dilution optimization experiment was done using a Human Cytokine/Chemokine Magnetic Bead Panel III kit (HCYP-3MAG-63K-11), containing 11 analytes, purchased from Merck Millipore. Briefly, pooled plasma samples were used to perform the optimization experiment, with five different combinations of diluted reagents as shown in the table 2.4 Experiment ("As recommended) was performed according to the recommendations of the manufacturer.

Table 2.4: Experiment conditions used for the Luminex reagent dilution optimization experiment

Experiment/Condition	Beads	Streptavidin-PE	Detection Antibody
1	As recommended	As recommended	As recommended
2	1:2 Dilution	1:2 Dilution	Undiluted (12.5µl)
3	1:3 Dilution	1:3 Dilution	Undiluted (12.5µl)
4	1:3 Dilution	1:3 Dilution	1:2 Dilution (25µl)
5	1:3 Dilution	1:3 Dilution	1:3 Dilution (25µl)

2.8.1.1 Results

There were significant differences between experiments 3 to 6 and the manufacturers recommended assay conditions (experiment 1) (Figure 2.2). As there was no significant difference between the manufacturer's recommendation (experiment 1) and experiment 2 (dilution of all reagents by 2), experiment 2 was used for all the Luminex experiments presented in this thesis. It is important to note that all quality control reagents yielded the required results when evaluated on kits using experiment 2 procedure. Implementation of this procedure leads to about cost savings of almost 50%.

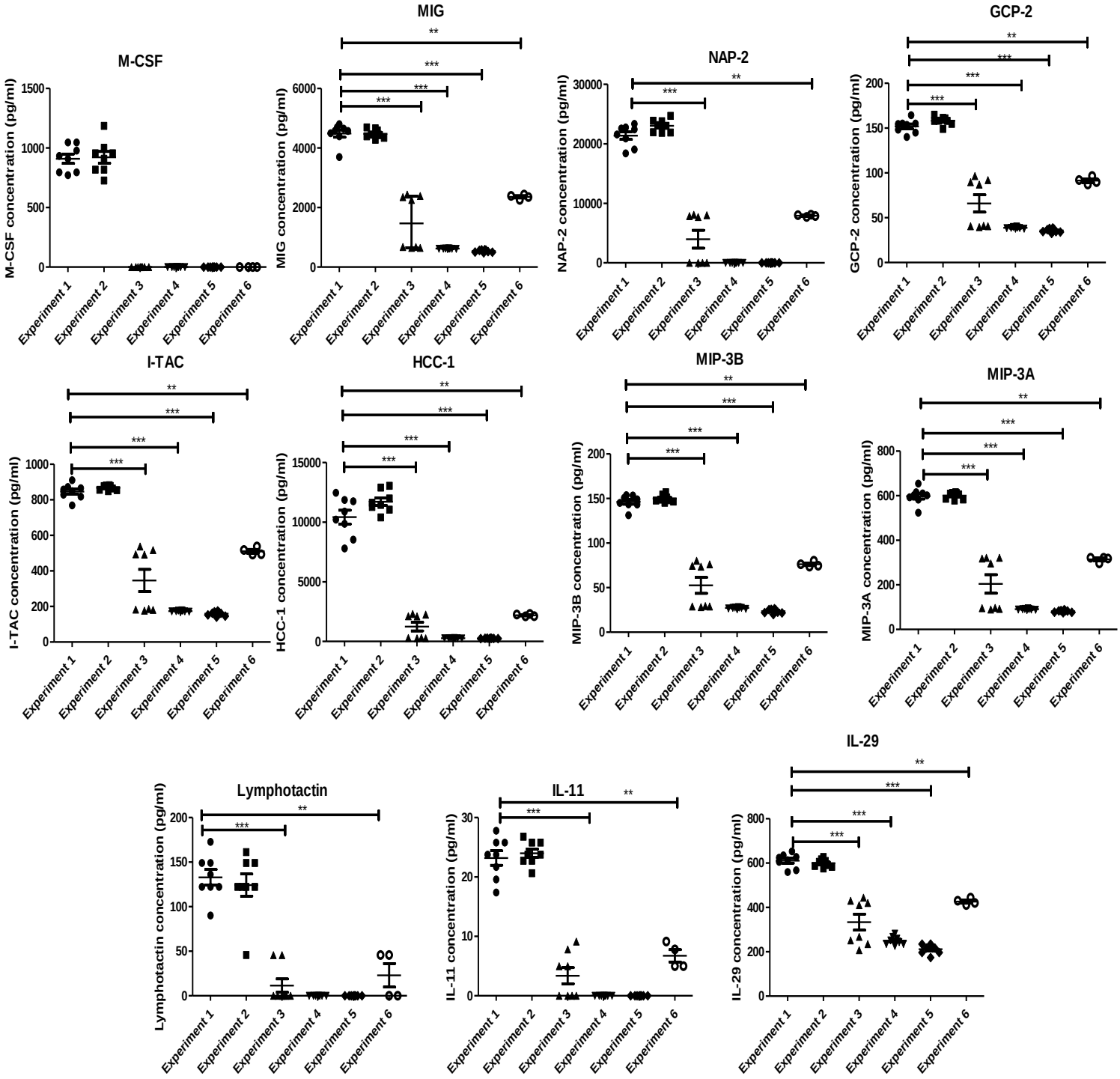

Figure 2.2: Optimization results comparing the different Luminex reagent dilution conditions. Differences between experiment 1 (recommended condition) and all the other conditions were analysed using the Mann-Whitney U test. **p value <0.05, *** p value <0.001.

2.8.2 Comparison of the Concentrations of Biomarkers Detected by the Luminex and Meso Scale Discovery Platforms

The two Luminex equipment in our laboratory (the Bio-Plex 200 system and Bio- Plex Magpix) are well established platforms that have been used for biomarker research in our group for several years. In addition to correlating with each other, results obtained with either of the Luminex instruments compare excellently with results generated from the same samples using different Luminex instruments in different laboratories all over the world. These findings are the result of the laboratory's ongoing participation in a Luminex External Quality Assurance Program (EQAPOL) which is coordinated by Duke University Vaccine Institute, with funding from the National Institutes of Health (NIH).

The Meso Scale Discovery (MSD) platform is a new platform that was recently acquired by our laboratory. As the MSD platform is a relatively new platform in the field of biomarker discovery, not much information is yet available in literature on the platform and no External Quality Assurance (EQA) scheme currently exists, that makes use of the platform. While analysing the plasma samples investigated in chapter 3 using the Luminex technology, we simultaneously investigated the levels of some of the biomarkers using the MSD platform. We therefore aimed to compare the results obtained from the established Luminex platform with results obtained from the MSD platform, in order to demonstrate that the new platform (MSD) was fit for purpose. We therefore hypothesized that although the absolute concentrations may not be the same, there was excellent correlation between cytokine measurements detected by the two platforms, which would enable usage of the MSD platform in future biomarker discovery and/or validation experiments.

Prior to the commencement of the experiments, plasma samples from all study participants were thawed on ice, aliquoted and re-frozen at -80^{O}C. On the date of the experiments, aliquots reserved for the different platforms (MSD or Luminex) were thawed, followed by the performance of experiments as recommended by the different manufacturers (Merck Millipore, for Luminex kits, and MSD for MSD assays) and reading on the different platforms following the settings recommended by the different manufacturers. We therefore compared the levels of seven proteins namely; CRP, SAA, IFN-γ, IL-1β, IL-13, TNF-α and MMP- 9 that were available for the two platforms.

2.8.2.1: Results

The descriptive statistical data obtained after comparing cytokine levels detected using the two platforms are shown in table 2.5

Table 2.5: Descriptive statistical analysis of data obtained when plasma samples were analysed using the Luminex and MSD platforms

	CRP (ng/ml)	CRP (MSD) (ng/ml)	SAA (ng/ml)	SAA (MSD) (ng/ml)	TNF-a (pg/ml)	TNF-a (MSD) (pg/ml)	IFN-g (pg/ml)	IFN-g (MSD) (pg/ml)	IL-1b (pg/ml)	IL-1b (MSD) (pg/ml)	IL-13 (pg/ml)	IL-13 (MSD) (pg/ml)	MMP-9 (pg/ml)	MMP-9 (MSD) (pg/ml)
Number of values	70	70	70	70	70	70	70	70	70	70	70	70	70	70
Minimum	0.0000	0.0000	0.0000	0.1373	0.6100	0.6820	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	160400	36.02
25% Percentile	1683	411.3	2627	1010	6.590	2.447	2.713	11.03	0.0000	0.01619	0.0000	0.5422	324800	101000
Median	4549	1438	8305	1462	11.98	3.285	14.82	21.63	1.815	0.07432	0.0000	0.9620	484100	181700
75% Percentile	18140	2704	23140	1773	22.25	5.028	79.26	99.43	8.228	0.3347	9.990	1.740	707700	310600
Maximum	290300	124200	43000	2453	414.6	302.8	660.5	869.1	239.6	16.77	1251	15.70	2662000	931400
Mean	30220	4981	14470	1367	23.13	12.58	91.32	102.5	15.66	0.6421	54.61	1.433	606600	233100
Std. Deviation	59790	16810	15650	474.6	51.27	48.79	167.7	174.4	42.49	2.287	178.2	2.089	463600	185200
Std. Error	7146	2009	1871	56.73	6.128	5.831	20.05	20.85	5.078	0.2733	21.30	0.2497	55410	22140
Lower 95% CI	15960	972.7	10740	1254	10.90	0.9470	51.32	60.95	5.530	0.09686	12.11	0.9351	496000	189000
Upper 95% CI	44470	8989	18200	1480	35.35	24.21	131.3	144.1	25.79	1.187	97.10	1.931	717100	277300

pg/ml, pico grams per millilitre ,ng/ml, nano grams per millilitre, Std Standard Deviation , CI ,Confidence Interval

We later evaluated the correlation between pairs of values obtained for each of the seven proteins using the Spearman correlation coefficient. Out of the seven host markers evaluated, only three (SAA, TNF-α and MMP-9) correlated well between the two platforms. Before and after graphs mapping the value obtained for each protein using the Luminex platform to the value obtained using the MSD platform are shown in figure 2.3.

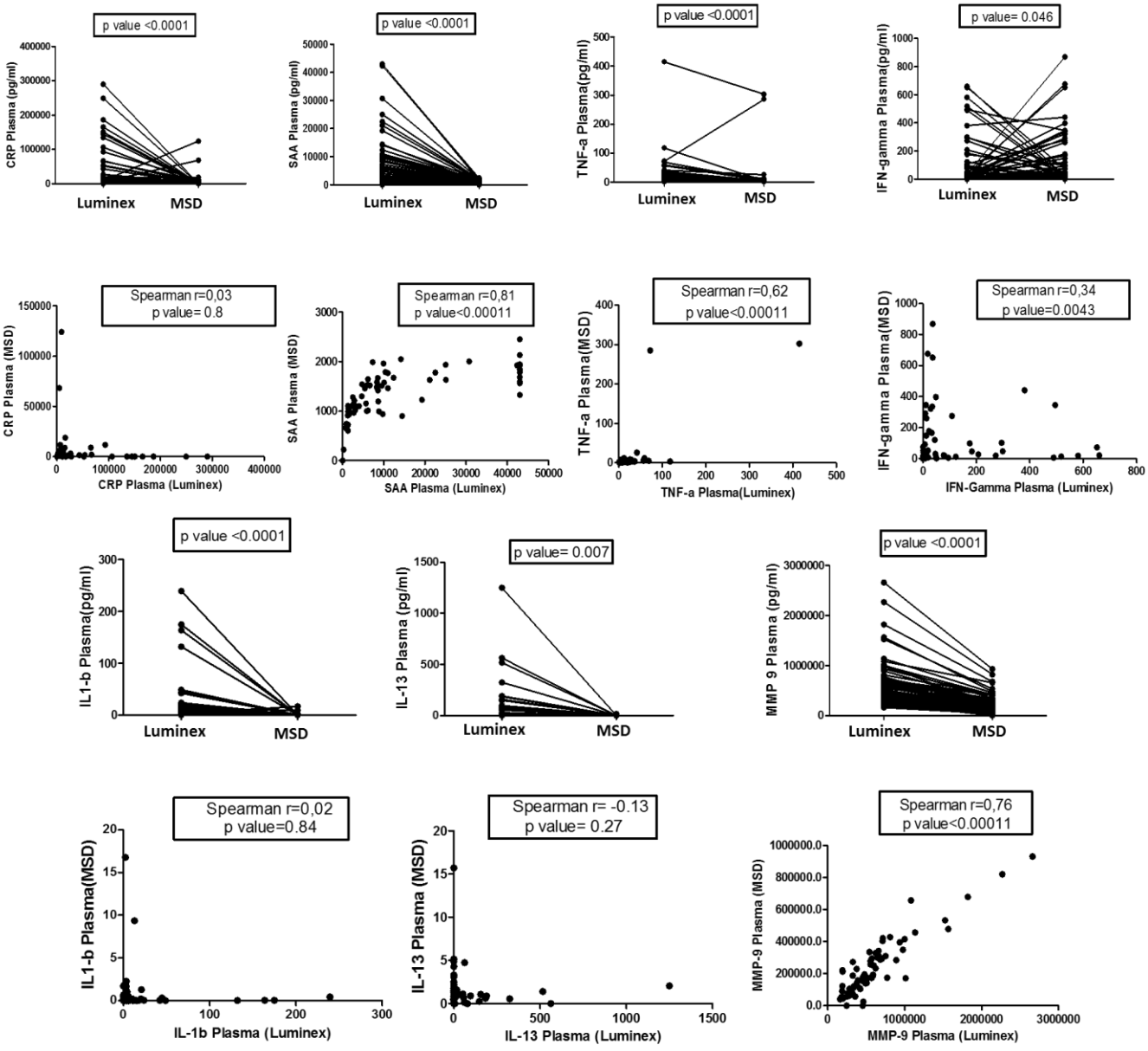

Figure 2.3: Before and after graphs showing the concentration of each analyte as measured by each instrument. The concentration of each analyte obtained from the Luminex instrument was mapped to the concentration of the same analyte in the same sample, as measured by the MSD instrument. The p-values in the before and after graphs

were calculated using the Mann Whitney U test whereas Spearman correlation coefficients and p-values are shown in the correlation graphs.

2.8.2.2: Discussion and Conclusion

It was not expected that the magnitude of responses detected by the two assays would be the same. However, it was expected that there would be an excellent correlation between the two platforms. Our data shows that results obtained between the two platforms do not correlate well, at least for the biomarkers investigated, in plasma samples. Therefore the implications are that if planning to use the MSD platform in future experiments, pilot experiments should be done prior to large experiments to ascertain if the markers of interest correlate with other well established platforms. It must be stressed that after completion of these experiments, the results were discussed with the manufacturer of the MSD platform, who evaluated the raw data, including data for standards and controls and confirmed that the results were valid. There reasons for the differences between the two platforms are not clear. However, the observed differences might be due to the different antibody epitopes or splice variants being measured in the kits supplied by the two companies. There is therefore a need for further investigation of the observed differences between the two platforms. As the Luminex platform is well-established and has been shown to correlate well with ELISA in experiments performed in our laboratory, and other laboratories worldwide, this platform was chosen for use in all the biomarker discovery experiments reported in this thesis.

2.8.3 ELISA Optimization Experiment

Prior to performing the ELISA experiments reported in Chapter 5, the kits were optimized for different sample dilutions and volumes. As the ELISA kit employed in the study were relatively new kits, which were still under development by the manufacturer, who was a collaborator on the project. Therefore it was deemed necessary that the assay be optimized, prior to use on the samples used in the study. The sample dilution and amount of sample used in the ELISA

experiments were therefore optimized prior to performing the required experiments as follows:

Samples from four TB patients and four latently infected individuals (as defined by a positive QuantiFERON in Tube test result) were evaluated on each optimization plate. Samples were pre-diluted (1:5) in glycerol and frozen at -80°C. On the day of the optimization experiment, samples were thawed and either further diluted 1:200 or 1:100 (in assay buffer), followed by the addition of 100µl or 200µl into the assay plate as shown in the plate layout below. Optimization experiments were done for Ig A and/or Ig G kits coated with "Kit 1", "Kit 2", LAM and thiol peroxidase (TpX) antigens.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A	BLANK	BLANK	LTBI 1	LTBI 1	LTBI 1	LTBI 1	LTBI 1	LTBI 1	LTBI 1	LTBI 1
B	Standard 1	Standard 1	LTBI 2	LTBI 2	LTBI 2	LTBI 2	LTBI 2	LTBI 2	LTBI 2	LTBI 2
C	Standard 2	Standard 2	LTBI 3	LTBI 3	LTBI 3	LTBI 3	LTBI 3	LTBI 3	LTBI 3	LTBI 3
D	Standard 3	Standard 3	LTBI 4	LTBI 4	LTBI 4	LTBI 4	LTBI 4	LTBI 4	LTBI 4	LTBI 4
E	Standard 4	Standard 1	TB 1	TB 1	TB 1	TB 1	TB 1	TB 1	TB 1	TB 1
F			TB 2	TB 2	TB 2	TB 2	TB 2	TB 2	TB 2	TB 2
G			TB 3	TB 3	TB 3	TB 3	TB 3	TB 3	TB 3	TB 3
H			TB 4	TB 4	TB 4	TB 4	TB 4	TB 4	TB 4	TB 4
Dilution			**1 in 200**		**1 in 200**		**1 in 100**		**1 in 100**	
Sample volume			**100 µL**		**200 µL**		**100 µL**		**200 µL**	

Figure 2.4: Layout for ELISA optimization experiment showing the dilution of samples and the amount of each diluted sample added to the optimization plate. Four TB and four latently infected individuals were used for the optimization experiments. Each sample was added in duplicate for each dilution (1:100/1:200) and each sample volume (100µl/200µl). The 1:200 dilution, and addition of 100µl of diluted sample was recommended by the manufacturers.

2.8.3.1 Results and Conclusion

There were no significant differences between the results obtained with the different dilution experiment conditions (Figure 2.5). All the subsequent ELISA experiments conducted in this thesis were performed using the manufacturer's recommended dilution conditions (1:200 dilution of samples and addition of 100µl of the diluted samples into plates).

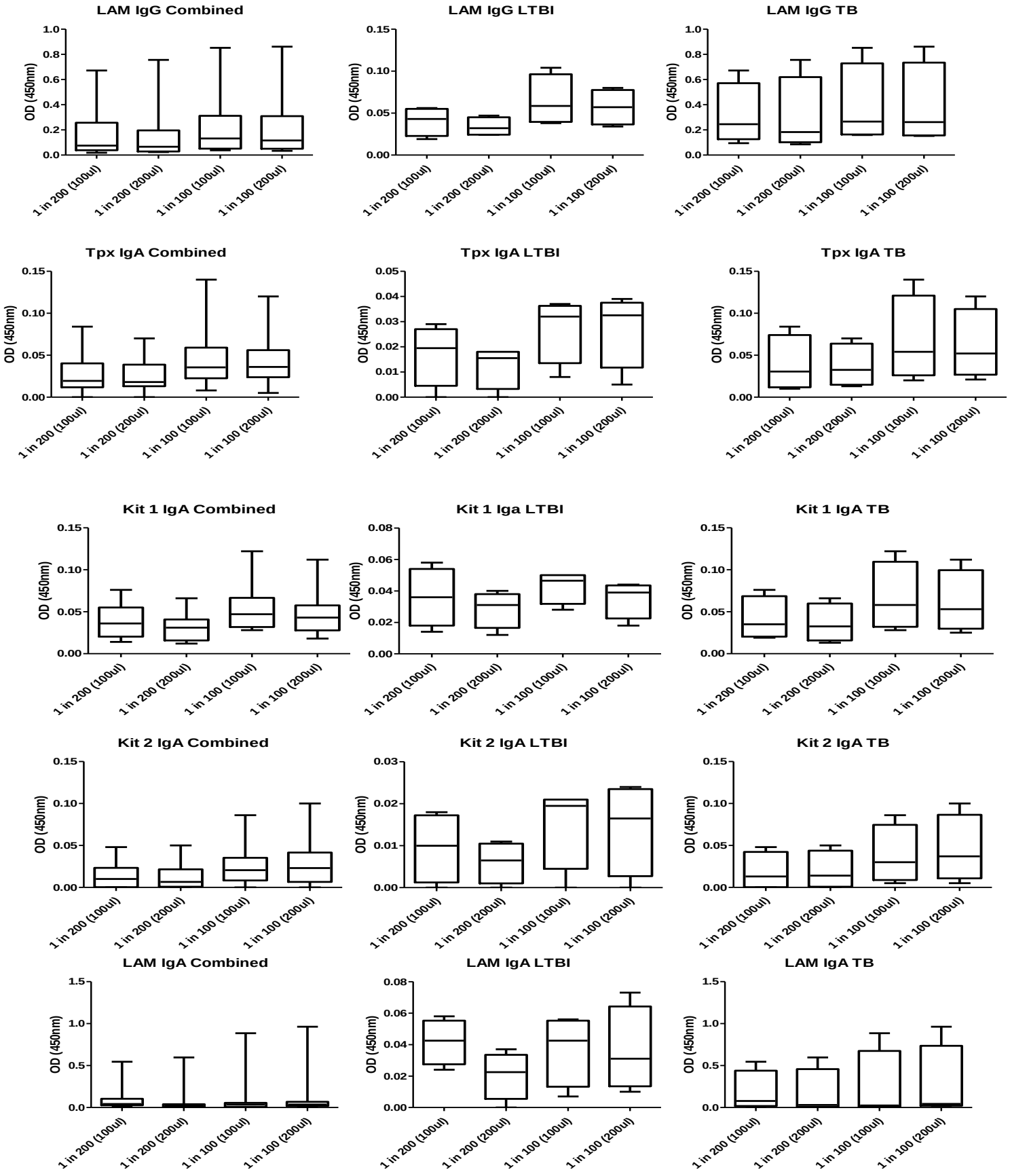

Figure 2.5: ELISA optimization results. The OD_{450nm} values are shown when the data generated from individuals with TB and LTBI was combined and OD_{450nm} values for TB and LTBI individuals were analysed separately. Differences between the recommended sample dilution and

volume (1:200 and 100µl) and all the other dilutions and volumes were analysed using the Mann-Whitney U test.

Chapter 3

Identification of novel host biomarkers in plasma as candidates for the immunodiagnosis of tuberculosis disease and monitoring of tuberculosis treatment response

Declaration:

The information reported in this chapter:

1) Was presented as a poster at the South African Immunology Society Conference, held in March 2016 at Glenburn Lodge, Muldersdrift, South Africa.

2) Was presented as a poster at the European Respiratory Society International Congress, held in September 2016 in London, United Kingdom.

3) Has been published in a peer reviewed Journal:

Reference: Jacobs R, Malherbe S, Loxton AG, Stanley K, van der Spuy G, Walzl G, Chegou N. **Identification of novel host biomarkers in plasma as candidates for the immunodiagnosis of tuberculosis disease and monitoring of tuberculosis treatment response.** Oncotarget. 2016 August 19; DOI: 10.18632/oncotarget.11420. PMID: 27557501

4) Has been provisionally patented:

Title: **Host Biomarkers for Immunodiagnosis and Monitoring of Tuberculosis Disease.** Inventors: **Jacobs R**, Chegou NN, Walzl G, Applicant: Stellenbosch University, Application type: Provisional Patent application, Country: South Africa, Application **No: 2016/02557,** Filling date: 15/04/2016.

3.1 Introduction

Tuberculosis (TB) disease, although curable, still accounted for the deaths of 1.5 million people in 2014 [9]. Rapid and accurate tools are urgently needed for early diagnosis of the disease, and monitoring of the response to treatment. The gold standard test for TB (culture) is not widely available, especially in resource-poor settings. The Ziehl-Neelsen sputum smear test is often the only available diagnostic tool in these settings, even though its limitations are well publicised [59]. Month 2 culture conversion is the most investigated biomarker for TB treatment response, but the wide unavailability of culture and its long turn-around time are serious limitations. Smear microscopy is not very useful for monitoring anti-TB treatment response as it is unable to distinguish live from dead bacilli [99]. The development of the geneXpert MTB/RIF test (Cepheid Inc., Sunnyvale, USA) was a significant advance in the TB diagnostic field as the test yields results within 2 hours, coupled with the detection of resistance to rifampicin, as a proxy for multi-drug resistant TB [63]. The high operating costs and need for infrastructure are amongst the major obstacles for its implementation in resource-poor settings. The geneXpert test is also not useful in monitoring of TB treatment response as the test cannot distinguish between DNA from dead and live bacteria [97]. Immunodiagnostic techniques may be useful in both the diagnosis of TB disease and monitoring of the response to treatment, especially as they may be easily adaptable into rapid, point-of-care tests, which would be suitable in resource-constrained settings. Furthermore, such tests will also be beneficial in cases where a conventional sputum-based diagnosis (smear microscopy, culture or geneXpert) is difficult e.g. in paediatric TB and in individuals with extrapulmonary TB.

IFN-gamma (IFN-γ) release assays (IGRA) remain the most widely used commercial immunodiagnostic tests for TB. These assays have been shown to be useful in the diagnosis of infection with *Mycobacterium tuberculosis (M.tb)* but as they cannot discriminate between active TB disease and latent *M.tb* infection, they are of limited value in high TB-endemic areas. The use of IGRAs as tools for monitoring of the response to TB treatment has so far yielded conflicting results [96,95]. An important limitation of overnight culture-based assays such as IGRAs is the fact that they are not suitable as point-of-care tests. These tests are therefore

not ideal for resource-constrained settings. The potential value of diagnostic approaches that are based on the detection of host biomarkers *ex vivo*, in easily obtainable samples such as saliva, serum or plasma has been demonstrated in previous studies [82–84, 100]. Such host biomarker based tests in addition to being used as diagnostic tests for TB, may also be useful in monitoring of the response to TB treatment [83,98,101]. Despite the promise so far shown in these previous investigations, no validated diagnostic tests based on the detection of host biomarkers in unstimulated samples currently exist. It is therefore important to validate the potential biomarkers that have so far been identified, and also identify new candidate host markers that might be useful in conjunction with previously identified biosignatures, in the diagnosis of TB disease.

In the present study, we investigated multiple host markers, including previously identified promising host markers, and relatively new markers, some of which have not previously been investigated in the TB field, as candidates for the immunological diagnosis of TB disease and monitoring of the response to TB treatment.

3.2 Materials and Methods

3.2.1 Study Participants

As discussed in chapter 2 (section 2.1),participants enrolled into the present study were individuals who presented with signs and symptoms requiring investigation for TB disease at the Fisantekraal Community Clinic in the outskirts of Cape Town, South Africa. The study was a sub-study of a larger diagnostic biomarker project (the African European Tuberculosis Consortium), that was ongoing at the study site and at field sites situated in six other African countries (www.ae-tbc.eu). All study participants presented with persistent cough lasting ≥2 weeks and at least one of either fever, malaise, recent weight loss, night sweats, knowledge of close contact with a TB patient, haemoptysis, chest pain or loss of appetite. Participants were eligible for the study if they were 18 years or older and willing to give written informed consent for participation in the study, including consent for HIV testing. Patients were excluded if they were pregnant, had not been residing in the study community for more than

3 months, were severely anaemic (haemoglobin <10 g/l), were on anti-TB treatment, had received anti-TB treatment in the previous 90 days or if they were on quinolone or aminoglycoside antibiotics during the past 60 days. The study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch.

3.2.2 Sample Collection and Diagnostic Tests

As discussed in Chapter 2 (section 2.2.1), 6ml of blood was collected at enrolment into heparinized BD vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) and transported to the laboratory at 4-8^{O}C for further processing. Upon receipt in the laboratory, tubes were centrifuged at 2000 rpm for 10 minutes after which plasma was harvested, aliquoted and stored at -80 ^{O}C until analysed. As discussed in chapter 2 (section 2.3), sputum samples were collected from all study participants and cultured using the MGIT method (BD Biosciences). Positive MGIT cultures were examined for acid fast bacilli using the Ziehl-Neelsen technique (to check for contamination), followed by Capilia TB testing (TAUNS, Numazu, Japan), to confirm the isolation of organisms of the *M.tb* complex, before being designated as positive cultures.

3.2.3 Classification of Study Participants and Reference Standard

As previously described in chapter 2 (section 2.3), participants were classified as definite TB cases, probable TB cases, participants with other respiratory diseases (ORD) or questionable disease status using a combination of clinical, radiological, and laboratory findings [83]. However, only definite TB cases (culture positive individuals) and those with ORD were included in this discovery study. Briefly, individuals with ORD had a range of other diagnoses, including upper and lower respiratory tract infections (viral and bacterial infections, although attempts to identify organisms by bacterial or viral cultures were not made), and acute exacerbations of chronic obstructive pulmonary disease or asthma. Because of the disproportionately high number of individuals with ORD, we included all the 22 culture

positive TB cases that were available at the study site and randomly selected 33 individuals with ORD from the study biobank, for inclusion into the current study.

3.2.4 Luminex Multiplex Immunoassay

The concentrations of 74 host markers including alpha-2-macroglobulin (A2M) , haptoglobin, C-reactive protein (CRP), serum amyloid P (SAP), procalcitonin (PCT), ferritin, tissue plasminogen activator (TPA), fibrinogen, serum amyloid A (SAA) (kits purchased from Bio-Rad Laboratories, Hercules, CA, USA), vitronectin, extracellular matrix protein 1 (ECM1), antithrombin III, vitamin D binding protein (VDBP), sFas, granzyme A, sFasL, sCD137, granzyme B, perforin, myoglobulin, ADAMTS13, P-selectin, lipocalin-2, growth differentiation factor (GDF) -15 , thrombopoietin (TPO), stem cell factor (SCF), B-cell attracting chemokine (BCA)-1, epithelial neutrophil activating protein (ENA-78), thymic stromal lymphopoietin (TSLP), I-309(CCL-1), stromal cell derived factor-1 alpha (SDF-1α), IFN-γ, IFN-α2, interferon gamma inducible protein (IP)-10 (CXCL10), macrophage inflammatory protein (MIP)-1β, tumor necrosis factor (TNF)-α, TNF-β, vascular endothelial growth factor (VEGF), soluble CD40 ligand (sCD40L), apolipoprotein (Apo) A-1, Apo CIII, complement component 3 (CC3), transthyretin, complement factor H (CFH), total plasminogen activator inhibitor-1 (PAI-1), neural cell adhesion molecule (NCAM), brain-derived neurotrophic factor (BDNF), cathepsin D, myeloperoxidase (MPO), matrix metalloproteinase (MMP)-2, MMP-9, monokine induced by gamma interferon (MIG/CXCL9), granulocyte chemotactic protein-2 (GCP2), interferon inducible T-cell alpha chemoattractant (I-TAC/CXCL11), hemofiltrate CC chemokine-1 (HCC1), α1-antitrypsin, pigment epithelium derived factor (PEDF), macrophage inflammatory protein-4 (MIP-4/CCL18), complement C4, interleukin (IL)-17F, IL-17A, IL-22, IL-33, IL-21, IL-23, IL-25, IL-31, IL-28A, IL-16, IL-1β, IL-12(p40), IL-13, IL-11 and IL-29 (kits purchased from Merck Millipore, Billerica, MA, USA), were investigated in plasma samples from all the study participants. The experiments were performed blindly, according to the instructions of the kit manufacturers, on the Bio-Plex platform (Bio-Rad). The Bio-Plex manager Software version 6.1 was used for bead acquisition and analysis of median fluorescence intensities.

3.2.5 Statistical Analysis

As discussed in chapter 2 (section 2.7), differences in the concentrations of host markers between TB patients and individuals with ORD were analysed using the Mann-Whitney U test. The diagnostic abilities of individual host markers were assessed by receiver operator characteristics (ROC) curve analysis. Optimal cut-off values and associated sensitivity and specificity were determined based on the Youden's Index [102]. The predictive abilities of combinations of host markers were investigated by general discriminant analysis (GDA), with leave-one-out cross validation [103]. Differences in the expression profiles of host markers during the course of TB treatment were analysed using mixed model repeated measures analysis of variance (ANOVA), with Fisher's Least Significant Difference (LSD) post hoc testing. P-values ≤0.05 were considered significant. The data were analysed using Statistica (Statsoft, Ohio, USA) and Graphpad Prism version 5 (Graphpad Software Inc., CA, USA).

3.3 Results

A total of 55 study participants, 22 of whom were culture positive TB patients were investigated in this study. The mean age of all study participants was 35.8 ± 10.2 years and 14 (25%) were HIV infected. The clinical and demographic characteristics of study participants are shown in table 3.1.

Table 3.1: Clinical and demographic characteristics of study participants. All the 22 TB patients included in the study were culture positive

Number of participants	All (n=55)	TB (n=22)	ORD (n=33)
Males, n (%)	22 (40)	7 (32)	15 (45)
Mean age, (Years)±SD	35.8 ± 10.2	38.8 ±10.1	33.9 ± 9.9
HIV Infected, n(%)	14(25)	4(18)	10(30)
Quantiferon results			
Positive, n (%)	34 (64)	15 (75)	19 (58)
Negative, n (%)	18 (34)	4 (20)	14 (42)
Indeterminate, n (%)	1 (2)	1 (5)	0 (0)

Abbreviations: TB= pulmonary tuberculosis, SD=standard deviation

3.3.1 Utility of individual host markers in the diagnosis of TB disease

When the baseline concentrations of host markers in TB patients (n=22) were compared to the levels detected in patients with ORD (n=33), by the Mann Whitney U test, the concentrations of 23 out of the 74 analytes were significantly different between the two groups. The median levels of CRP, SAP, PCT, ferritin, TPA, SAA, ADAMTS-13, p-selectin, GDF-15, I-309, IFN-γ, IP-10, TNF-α, CFH, MIG, ITAC, HCC-1 and MIP-4 were significantly higher in TB cases, whereas the levels of antithrombin III, Apo A-1, transthyretin, NCAM and BDNF were significantly higher in the ORD group. Trends ($0.05<p \leq 0.01$) towards higher levels of sFas, lipocalin-2, VEGF, PEDF, CC4 and IL-33 were observed in TB cases (Table 3.2). When the diagnostic accuracies of individual host markers were investigated by ROC curve analysis, the area under the ROC curve (AUC) was ≥0.70 for 18 markers (Table 3.2). The most accurate single host markers included CRP, SAP, NCAM, TPA, I-309, and MIG, which all performed with AUC ≥ 0.80 (Table 3.2). Representative plots showing some of the most accurate individual host markers are shown in Figure 3.1. When data was stratified according to HIV infection status, concentrations of three additional markers (A2M, MIP-1β and VEGF) became significant in the two groups, with AUC's of 0.70, 0.69 and 0.69 respectively.

Table 3.2: Median levels (and inter-quartile ranges in parenthesis) of host biomarkers detected in baseline plasma samples from pulmonary TB patients (n=22) and individuals with other respiratory diseases (n=33) and their diagnostic accuracies for TB disease. Only analytes showing significant differences or trends between groups with the Mann-Whitney U test are shown. Optimal cut-off values and associated sensitivity and specificity were determined based on the Youden's Index. The concentrations of CRP, SAP, SAA, antithrombin III, ADAMTS-13, p-selectin, GDF-15, Apo A-1, transthyretin, CFH, sFAS, lipocalin-2, MIP-4 and CC4 are in ng/ml. The concentrations of all the other analytes are in pg/ml.

Marker	ORD (n=33)	TB Disease (n=22)	P value	AUC (95% CI)	Cut-off value	Sensitivity % (95% CI)	Specificity % (95% CI)
ADAMTS-13	3297 (2569-4504)	4235 (2766-8073)	0.044	0.66 (0.51- 0.81)	> 3466	68 (45-86)	64 (45-80)
Antithrombin III	803100 (624200-968800)	625700 (519000-714300)	0.01	0.70 (0.56- 0.84)	<744162	91 (71-99)	61 (42-77)
Apo A-1	431400 (334100-548700)	274300 (242800-351500)	0.0014	0.76 (0.62- 0.89)	< 318930	73 (50-89)	82 (65-93)
BDNF	5774 (3824-8838)	3791 (1683-6187)	0.017	0.69 (0.55-0.84)	< 3467	45 (24-68)	91 (76-98)
CC4	114800 (70110-176600)	151600 (95100-291100)	0.100	0.63 (0.48- 0.79)	> 212263	32 (14-55)	97 (84-100)
CFH	729100 (557000-795100)	875400 (715200-980300)	0.0072	0.72 (0.57- 0.86)	> 808359	68 (45-86)	82 (65-93)
CRP	2019 (440-6330)	52980 (10020-137400)	P<0.0001	0.89 (0.79 -1.00)	> 9081	82 (60-95)	90 (76-98)
Ferritin	62850 (41840-120100)	161000 (116800-355300)	P<0.0001	0.78 (0.64 - 0.92)	> 93785	91 (71-99)	67 (48-82)
GDF-15	19.2 (9.8-41.7)	49.24 (25.10-125.5)	0.002	0.75 (0.62 - 0.88)	> 21.06	91 (71-99)	55 (36-72)
HCC1	108000 (73120-130200)	144100 (108500-171800)	0.0022	0.75 (0.61- 0.89)	> 136956	59 (36-79)	85 (68-95)
I-309	1.24 (1.1-1.4)	2.25 (1.4-3.5)	0.0002	0.80 (0.67- 0.93)	> 1.945	68 (45-86)	90 (73-98)
IFN-γ	5.78 (0.39-49)	31.06 (8.81-156)	0.02	0.69 (0.54 - 0.83)	> 3.910	91 (70-99)	48 (31-66)
IL-33	88.77 (21.75-211.5)	164.9 (70.79-251.9)	0.100	0.63 (0.48- 0.78)	> 131.8	68 (45-86)	61 (42-77)
IP-10	444 (258-876)	1469 (878-3865)	P<0.0001	0.78 (0.64 - 0.91)	> 746.6	86 (65-97)	73 (54-87)

ITAC	628.0 (87.49-1253)	1106 (519.1-2042)	0.022	0.68 (0.54-0.83)	> 276.5	95 (77-100)	36 (20-55)
Lipocalin-2	453.9 (300.5-567.3)	600.7 (346.7-1028)	0.062	0.65 (0.50- 0.80)	> 552.8	59 (36-79)	76 (58-89)
MIG	312.4 (87.21-1028)	3076 (592.2-13830)	P<0.0001	0.81 (0.69- 0.94)	> 1700	68 (45-86)	88 (72-97)
MIP-4	92.5 (53.6-152)	208 (90-369)	0.012	0.70 (0.55- 0.85)	> 220.9	50 (28-72)	91 (76-98)
NCAM	592100 (430200-684200)	350800 (306800-421000)	P<0.0001	0.88 (0.78- 0.98)	< 477229	91 (71-99)	73 (54-87)
PCT	7520 (6749-8370)	8702(8185-9888)	0.0009	0.77 (0.64- 0.90)	> 8101	86 (65-97)	67 (48-82)
PEDF	10790 (8852-12870)	12360 (10360-15270)	0.0502	0.66 (0.50- 0.81)	> 11423	68 (45-86)	64 (45-80)
p-selectin	202 (163-549)	441 (263-796)	0.030	0.67 (0.53 - 0.82)	> 265.7	77 (55-92)	58 (39-75)
SAA	5972 (1324-12570)	9837 (6078-43000)	0.0081	0.71 (0.58 - 0.85)	> 8626	68 (45-86)	70 (51-84)
SAP	21850 (16980-24670)	30660 (23820-45050)	P<0.0001	0.85 (0.72-0.98)	> 25958	68 (45-86)	85 (68-95)
sFAS	5.3 (1.8-8.0)	8.03 (4.52-13.22)	0.065	0.65 (0.50- 0.81)	> 6.7	67 (43-85)	68 (49-83)
TNF-a	7.4 (4.1-13.4)	15.9 (11.8-24.6)	0.0024	0.74 (0.61- 0.88)	> 10.85	82 (60-95)	73 (54-87)
TPA	5895 (5187-6507)	7199 (6536-7702)	0.0002	0.80 (0.68- 0.92)	> 6307	86 (65-97)	76 (58-89)
Transthyretin	544700 (398000-638500)	293700 (212700-397500)	0.0005	0.78 (0.65- 0.91)	< 416242	82 (60-95)	76 (58-89)
VEGF	147 (0-546)	289 (134.3-877)	0.081	0.64 (0.50- 0.79)	> 175.6	73 (50-89)	55 (36-72)

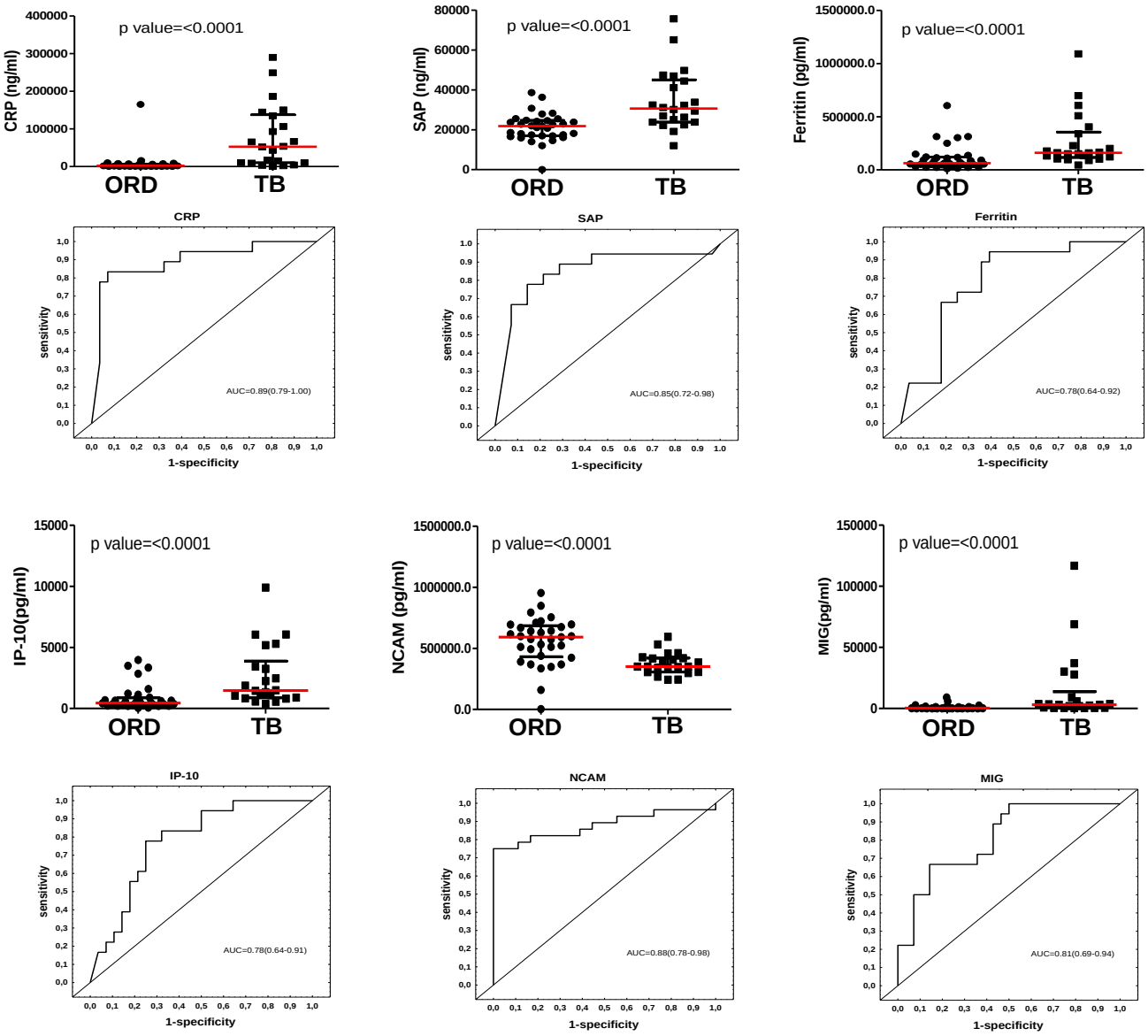

Figure 3.1: Concentrations of host markers detected in plasma samples from TB patients (n=22) and individuals with other respiratory diseases (n=33) and receiver operator characteristics curves showing the accuracies of these markers in the diagnosis of TB disease. Representative plots are shown for CRP, SAP, ferritin, IP-10, NCAM and MIG. Error bars in the scatter dot plots represent the median with interquartile range.

3.3.2 Utility of Multi-Plasma Marker Biosignatures in the Diagnosis of TB Disease

When the data obtained from all the TB patients and those with ORD were fitted into General Discriminant Analysis (GDA) models regardless of HIV status, combinations between up to six

different host markers showed potential in the diagnosis of TB disease. A five-marker biosignature comprising of NCAM, SAP, ferritin, CFH and ECM-1 diagnosed TB disease with a sensitivity of 95.2% (95% CI, 81.0-99.9%) and specificity of 92.9% (95% CI, 70.8-98.9%) in the resubstitution classification matrix and sensitivity of 95.2% (95% CI, 81.0-99.9%) and specificity of 89.3% (95% CI, 66.4-97.2%) after leave-one-out cross validation. However, the most optimal diagnostic biosignature irrespective of HIV status was a combination between six markers (NCAM, SAP, IL-1β, sCD40L, IL-13 and Apo A-1), which diagnosed TB disease with a sensitivity 100% (95% CI, 86.3-100%) and specificity of 89.3% (95%CI, 67.6-97.3%) after leave-one-out cross validation. The positive and negative predictive values of the six-marker biosignature were 87.5% (95% CI, 66.5-96.7%) and 100% (95%CI, 83.4-100%) respectively (Figure 3.2, Table 3.3).

When the GDA procedure was repeated after excluding the HIV infected individuals, two six-marker biosignatures:- NCAM, A2M, IL-22, ferritin, myoglobulin and IL-12(p40), and NCAM, A2M, IL-22, ferritin, TNF-β and MIP-4, diagnosed TB disease with both sensitivity and specificity of 100% (AUC = 1.0, 95% CI, 1.0-1.0) (Table 3.3). NCAM was the most frequent analyte in biosignatures, appearing in all the top 13 biosignatures for diagnosing TB disease regardless of HIV infection status, and in 68%(23 of the 34) biosignatures that were generated for the diagnosis of TB disease after excluding HIV infected individuals. Other markers that occurred most frequently in diagnostic biosignatures for TB disease included GDF-15, SAP, CFH, A2M, TNF-β, ferritin, SDF-1α amongst others (Figure 3.2).

Table 3.3: Accuracies of plasma protein biosignatures in the diagnosis of TB disease. Only the top two biosignatures generated for the diagnosis of TB disease, regardless of HIV infection status and in the HIV uninfected individuals only, are shown. The importance of the different analytes in biosignatures for the diagnosis of TB disease is shown in Figure 3.2.

Biosignature	**Resubstitution Classification matrix**				**Leave-one-out cross validation**			
	Sensitivity % (95% CI)	**Specificity % (95%, CI)**	**PPV % (95% CI)**	**NPV % (95 % CI)**	**Sensitivity % (95% CI)**	**Specificity % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**
Accuracy of biosignatures regardless of HIV infection status								
NCAM+ SAP+ ferritin+ CFH+ECM-1	95.2 (81.0-99.9)	92.9 (70.8-98.9)	90.9 (69.4-98.4)	96.3 (79.1-99.8)	95.2 (81-99.9)	89.3 (66.4-97.2)	87 (65.3-96.6)	96.2 (78.4-99.8)
NCAM+ SAP+IL-1β+sCD40L +IL-13+Apo A-1	100 (86.3-100)	89.3 (67.6-97.3)	87.5 (66.5-96.7)	100 (83.4-100)	100 (86.3-100)	89.3 (67.6-97.3)	87.5 (66.5-96.7)	100 (83.4-100)
Accuracy of biosignatures in HIV uninfected individuals								
NCAM+A2M +IL22+ ferritin+ myoglobulin +IL-12(p40)	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)
NCAM+A2M +IL22+ ferritin+ TNF-β+MIP-4	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)	100 (78.1-100)	100 (79.1-100)

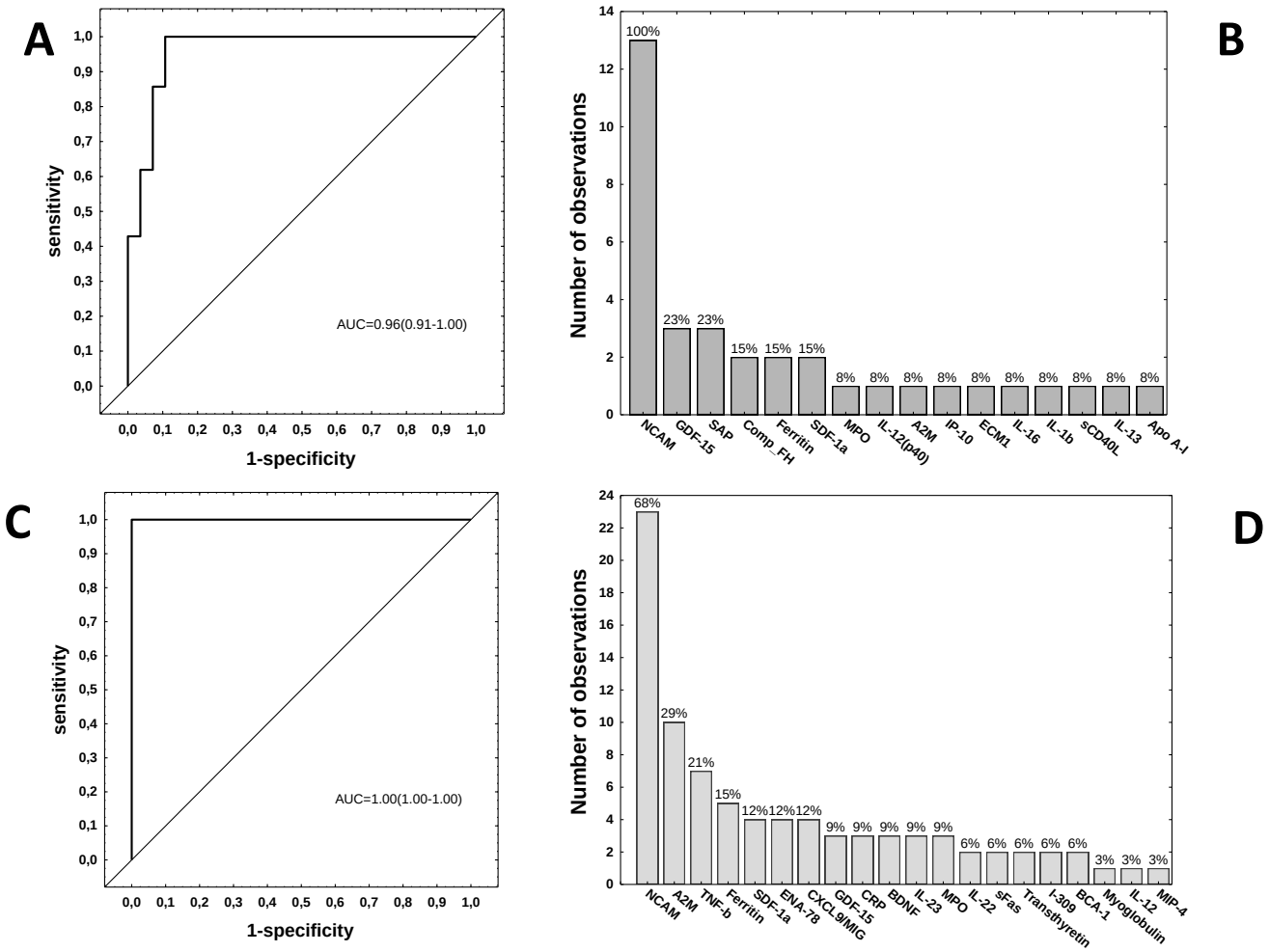

Figure 3.2: Accuracy of multi-marker models in the diagnosis of TB disease. Receiver operator characteristics (ROC) curve showing the accuracy of the most accurate six-marker biosignature (NCAM, SAP, IL-1β, sCD40L, IL-13 and Apo A-1) in the diagnosis of TB disease in all study participants, regardless of HIV infection status (A), frequency of analytes in the top 13 general discriminant analysis (GDA) models that most accurately classified study participants as TB disease or ORD irrespective of HIV status (B), ROC curve showing the accuracy of the most accurate six-marker biosignature (NCAM+A2M+IL-22+ferritin+ myoglobulin+IL-12(p40) or NCAM+A2M+IL-22+ferritin+TNF-β+MIP-4) in the diagnosis of TB disease in HIV negative study participants (C), and frequency of analytes in the top 34 GDA models that most accurately classified study participants as TB disease or ORD in the absence of HIV infection (D). The bar graphs (B and D) indicate the frequency of analytes in the most accurate GDA models.

3.3.3 Changes in Host Biomarker Levels during the Course of TB Treatment

To investigate whether any of the 74 host markers could potentially be used to monitor the response to TB treatment, the host markers were evaluated in plasma samples that were collected from TB patients at the end of standard TB treatment (month 6). Of the 22 TB patients that were investigated in this study however, only 15 (68%) returned to the clinic and provided samples at the end of treatment. Compared to baseline levels, the concentrations of 11 host markers changed significantly during the course of treatment. There was a significant decrease in the levels of CRP, SAP, ferritin, IFN-γ, VEGF, IP-10, CC3, CFH and α-1-antitrypsin from baseline to month 6, whereas a significant increase in the levels of transthyretin and MMP-2 was observed. The levels of IL-1β, SAA, sFas and MIG showed trends towards decreasing levels from baseline to month 6, whereas Apo-CIII, Apo A-1 and GCP-2 showed trends towards increasing levels at the end of treatment (Figure 3.3).

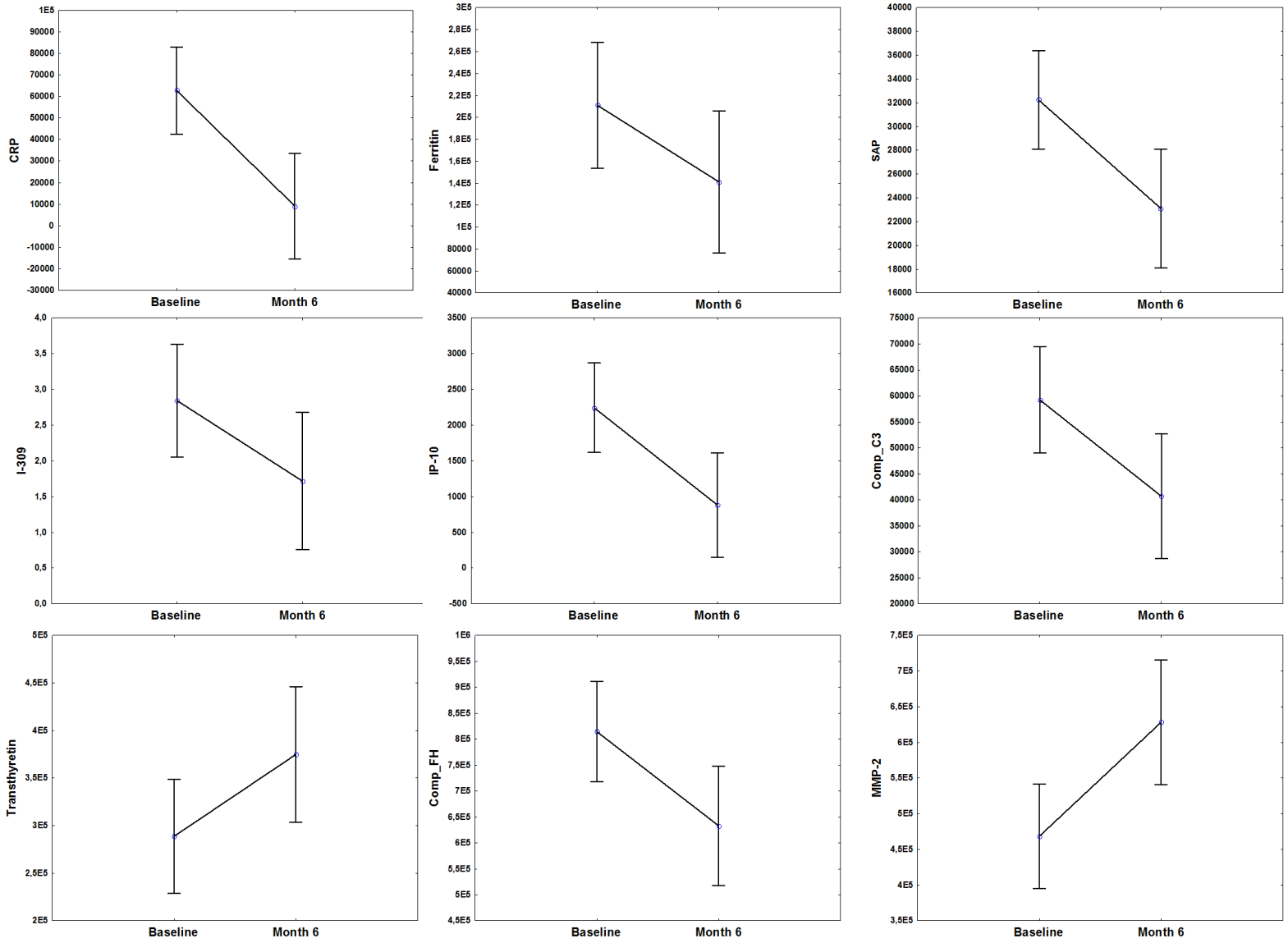

Figure 3.3: Before (baseline) and after treatment (month 6) concentrations of host markers in plasma samples from TB patients. Plasma was collected from patients at recruitment, prior to the initiation of anti-TB therapy and then at the end of standard TB treatment (month 6). Error bars indicate the Least Squared means with 95% Confidence Intervals.

3.4 Discussion

We investigated the diagnostic potentials of 74 host markers in plasma samples that were obtained from confirmed active TB cases and individuals with ORD, as candidates for the diagnosis of TB disease. Although 18 of the 74 host markers including relatively new

biomarkers in the TB field namely; antithrombin III, GDF-15, NCAM, HCC1, MIP-4 and recently identified markers I-309, MIG, Apo A-1, transthyretin and CFH, showed potential in the diagnosis of TB disease, regardless of HIV infection status as determined by area under the ROC curve (AUC), the most optimal diagnostic biosignature irrespective of HIV infection status was a six-marker model comprising of NCAM, SAP, IL-1β, sCD40L, IL-13 and Apo A-1, which diagnosed TB disease with a sensitivity of 100% and specificity of 89.3%, with promising positive and negative predictive values. In the absence of HIV infection, six-marker biosignatures diagnosed TB disease with 100% accuracy.

Amongst the 18 host markers that showed the most potential in the diagnosis of TB disease individually as determined by AUC, six (CRP, SAP, PCT, ferritin, TPA and SAA) were acute phase proteins, five (I-309, MIP-4, Apo A-1, transthyretin and CFH) were markers that play various roles in the body and have recently been identified as promising TB diagnostic candidates [104,82], two (TNF-α and IP-10) are widely investigated TB biomarkers [105] whereas four (antithrombin III, GDF-15, NCAM, HCC1), were relatively new markers, which have not previously been investigated in the TB field. Other host markers including ECM-1, IL-1β, sCD40L and IL-13 although not very promising individually in the current study, were included into the top diagnostic biosignatures for TB disease.

Acute phase proteins are primarily produced by the liver, and act as opsonins at inflammatory sites [106]. Serum CRP has been extensively studied and shown to be promising for both the diagnosis of TB disease and monitoring of TB treatment response [107,108]. SAP, a homologue of CRP, has also been shown to have a protective role against bacterial infections [109]. Ferritin is a well-recognised protein in iron storage processes. It has long been established that iron acquisition is an essential virulence mechanism for pathogenic bacteria [110,111]. The concentrations of all the acute phase proteins that showed potential in the current study were elevated in the TB patients, and this is in agreement with previous findings [111,82,112].

The chemokines; MIG(CXCL9), IP-10(CXCL10), ITAC(CXCL11) and I-309(CCL-1) are found in abundance in activated bronchial epithelium [113]. MIP-4(CCL18) is a chemokine that is produced mainly by antigen presenting cells [114]. These chemokines play vital roles in the

recruitment of activated T-cells to the site of infection [113][115]. Our finding of significantly higher levels of these chemokines in the plasma of patients with active TB is in agreement with previous observations [104,116,117,114]. The relatively new host markers investigated in the study (GDF-15, antithrombin III, HCC1 and NCAM) showed potential individually, in the diagnosis of TB disease. Other markers including p-selectin, ADAMTS-13 and BDNF although not amongst the most promising single markers as demonstrated by ROC curve analysis, were significantly different between the TB patients and individuals with ORD, with the Mann-Whitney U test. GDF-15 is a member of the transforming growth factor beta superfamily (TGF-β) and its expression is associated with tissue damage, but has also been reported to exhibit tissue protective functions [118,119]. It has been identified as a prognostic marker for prostate cancer, with high serum levels observed in patients with liver cirrhosis and hepatocellular carcinoma [120,121]. Antithrombin complexes are important mediators of the coagulation system, with antithrombin III being one of the most important inhibitors of this system [122]. Markedly lowered antithrombin III plasma levels have been observed in sepsis [123]. HCC1 has been identified as a monocyte chemoattractant, with high concentrations observed in patients with chronic renal failure [124]. P-selectin is part of the selectin family of cell adhesion molecules, that promotes inflammatory reactions [125]. ADAMTS-13 is a metalloprotease with thrombospondin repeats, and has shown low activity in patients with recurrent thrombaotic thrombocytopenic purpura [126,127]. In a previous study by Liu et al, GDF-15 levels were not significantly different between TB patients, latently infected individuals and healthy controls [121]. However, GDF-15 levels showed potential in the current study. In agreement with the findings of the current study, Mukae et al observed significantly higher levels of p-selectin in TB patients in comparison to individuals with ORD [128]. For the other newly identified TB diagnostic candidates, we observed higher levels of HCC1 and ADAMTS-13 in TB patients, but the levels of antithrombin III and NCAM were higher in individuals with ORD.

NCAM (CD56) is important in cell-cell or cell-matrix interactions, and is involved in neuronal differentiation, branching and survival [129]. It has been shown to play a role in lung tumor progression [130]. In the present study, we demonstrate for the first time, the potential of NCAM as a biomarker for TB disease. NCAM was the most frequently occurring marker in biosignatures for the diagnosis of TB disease, and was included in all the top 13 marker

combinations for the diagnosis of TB disease regardless of HIV infection status, and in 68% of the models that were generated when HIV infected individuals were excluded. The combination of NCAM with five other markers (SAP, IL-1β, sCD40L, IL-13 and Apo A-1), diagnosed TB disease regardless of HIV infection status, with high accuracy, whereas all the TB patients and individuals with ORD were accurately classified (100% sensitivity and specificity) when NCAM was used in combination with either A2M+IL-22+ferritin+myoglobulin+IL-12(p40), or A2M+IL-22+ferritin+TNF-β+MIP-4, in the absence of HIV infection. In our previous serum-based study [82], optimal diagnosis of TB disease was achieved using a seven-marker biosignature of CRP, IFN-γ, IP-10, Apo A-1, transthyretin, SAA and CFH. While our previous study was conducted on 716 participants that were recruited from five different African countries, the current study was conducted on 55 individuals who were recruited at a single study site, and analysis was performed on plasma and not serum samples. There was excellent agreement between the findings of this and the serum study, as the diagnostic potential observed for individual host markers including CRP, SAA, SAP, CFH, Apo A-1, transthyretin, ferritin amongst others, was replicated in the current study. This implies that the new host markers identified in this study may be used in conjunction with, or as alternatives to the markers that were included in the previous seven-marker biosignature [82] if a point-of-care screening test based on these biosignatures were to be developed. However, further studies are required to determine the best combination of analytes before such a screening test can be developed.

In addition to being potentially useful as diagnostic candidates for TB disease, 11 of the markers investigated including CRP, SAP, ferritin, IP-10, α-1-antitrypsin changed with treatment, thereby indicating that they may be potential candidates for monitoring of the response to TB treatment. Although our observations for MMP-2 (increasing levels from baseline to month 6) were contrary to the observations of Ugarte-Gil et al (decreasing levels from baseline to month 6 in sputum culture positive individuals after 2 weeks of treatment) [131], our observations for CRP, SAP, ferritin, IP-10, and α-1-antitrypsin are in agreement with findings from previous studies [132,133]. More studies are required to validate the potential of these biomarkers, and investigate whether any of the markers would be able to distinguish between clinical cure, treatment failure and relapse. Furthermore, it will be important that samples collected early after the initiation of TB treatment and regularly in the course of

treatment are included in such future studies, as biomarkers for early indication of the response to TB treatment are an urgent need worldwide.

The main limitation of the current study was the small sample size. In spite of this limitation, we confirmed the diagnostic potentials of host markers that were only recently identified as potential TB diagnostic candidates, identified new TB host biosignatures, and our study was done on individuals that presented with signs and symptoms, prior to the establishment of a clinical diagnosis, and at a community level health care clinic in a high-burden setting. Further studies are required to validate the new findings from our study preferably in larger numbers of study participants presenting with signs and symptoms requiring investigation for TB disease, and in studies conducted in other geographical regions. As the number of HIV infected study participants were limited in the current study, the biosignatures identified in this study require further investigation in HIV infected individuals. Importantly, HIV infected individuals included in such future studies should be staged with CD4 cell counts and viral loads so as to investigate the influence of severe HIV infection on the accuracy of the biosignatures. As we only investigated adult culture positive TB patients in the present study, the utility of the biosignatures also needs to be assessed in difficult to diagnose TB cases such as paediatric and extrapulmonary TB, smear and culture negative TB, and also in patients presenting with confirmed diseases that are similar to TB, including non-TB pneumonias. Validated biosignatures could then be incorporated into a point-of-care screening test for TB, preferably based on the lateral flow technology as recently demonstrated in a multi-centered African study [134].

In conclusion have identified candidate host markers, some of which have not previously been investigated in TB, as diagnostic candidates for TB disease. The biosignatures identified in our study require further validation in large-scale multi-site prospective studies.

Chapter 4

Diagnostic Potential of Novel Salivary Host Biomarkers as Candidates for the Immunological Diagnosis of Tuberculosis Disease and Monitoring of Tuberculosis Treatment Response

Declaration:

The information reported in this chapter:

1) Was presented as a poster at the South African Immunology Society Conference, held in March 2016 at Glenburn Lodge, Muldersdrift, South Africa.

2) Was presented as a poster at the European Respiratory Society International Congress, held in September 2016 in London, United Kingdom.

3) Has been published in a peer reviewed Journal:

Reference: Jacobs R, Maasdorp E, Malherbe S, Loxton AG, Stanley K, van der Spuy G, Walzl G, Chegou N. **Diagnostic Potential of Novel Salivary Host Biomarkers as Candidates for the Immunological Diagnosis of Tuberculosis Disease and Monitoring of Tuberculosis Treatment Response.** *PLOS ONE* 2016,doi:10.1371/journal.pone.0160546

4.1 Introduction

Tuberculosis (TB) remains a global health problem. According to the World Health Organisation (WHO), 1.5 million people died from the disease in 2014 [9]. The global TB epidemic continues to in part be driven by undiagnosed TB cases or delays in the diagnosis of the disease, which results in delays in treatment initiation and increases chances of transmission. Therefore the need for rapid and accurate tools for both the diagnosis and monitoring of TB treatment response remains a priority for the global control of the disease. Current diagnostic tools have several drawbacks, including the low sensitivity of the Ziehl-Neelsen smear microscopy test and the unavailability and long turn-around time of the current gold standard (culture). Furthermore the long turn-around time of culture limits its use as a means to monitor the response to TB treatment [59,99]. The diagnosis of TB disease has significantly improved with the roll-out of the automated gene amplification test GeneXpert (Cepheid Inc., Sunnyvale, USA), as the test greatly reduces the time to detection and is coupled with the identification of resistance to rifampicin. However this test is costly and requires infrastructure that is not readily available in resource constrained settings, and is therefore not ideal in these areas [62]. Immunodiagnostic approaches might be beneficial especially if based on more easily available sample types such as saliva, whole blood, plasma or serum, for both the diagnosis of TB disease and monitoring of treatment response. The relatively easier adaptability of host biomarker-based tests into rapid point-of-care tests, makes them very promising for resource-constrained settings [80]. Additionally, such tests may be useful particularly in circumstances where sputum collection is difficult, for example, in paediatric TB, and in paucibacillary forms of the disease such as extra-pulmonary TB and co-infection with HIV.

Interferon gamma (IFN-γ) release assays (IGRAs) and the tuberculin skin test remain the most widely used commercially available TB immunodiagnostic tests. The use of IGRAs is however limited in high TB endemic areas as these assays are not useful in the diagnosis of active TB disease, which is a major problem in these areas with high prevalence of latent infection [135]. IGRAs have also generated inconsistent results as tools for monitoring of the response to TB treatment [95,96]. Host markers other than IFN-γ detected after overnight stimulation with the antigens employed in IGRAs (ESAT-6/CFP-10/TB7.7) and markers produced after

stimulation with novel *M.tb* infection phase dependent antigens have shown promise [73, 81]. However overnight culture-based assays are unable to serve as rapid, point-of-care tests. Host biomarkers detected in *ex vivo* samples such as serum, plasma, saliva and other effusions have shown potential in the diagnosis of TB disease [83, 84, 136, 137]. Although saliva has been shown to be an important diagnostic fluid in numerous diseases, including systemic, oral infections, and HIV [138], not much has been done on this potentially valuable sample type in the TB field. Saliva is an easily obtainable sample and can be collected non-invasively with limited training and basic equipment. It is abundantly available in all individuals and an average adult has been reported to always have about 1ml of saliva in the oral cavity [139]. Recent studies have shown an up to 6-fold higher expression of some host biomarkers in saliva when compared to serum samples from TB patients [84], and that some of these host markers may be useful as tools for the diagnosis of TB disease and monitoring of the response to treatment [84,83]. Given the potential that diagnostic tools based on host biomarkers detected in saliva might have in the control of TB disease, it is important to continue investigating new host biomarkers in this potentially valuable sample type, in an attempt to identify better diagnostic candidates, and to refine the markers that have shown potential in previous studies, pending validation in larger prospective studies. In a recent large multi-centered study conducted on serum samples [82], combinations between new and established host biomarkers showed potential in the diagnosis of TB disease. As most of the new candidate biomarkers have not been previously investigated in saliva, we aimed to investigate the diagnostic utility of salivary levels of these host markers for TB disease and to ascertain whether any of these markers were potentially useful in monitoring of the response to TB treatment. We also investigated the utility of biomarkers that showed potential as salivary diagnostic candidates in recently published studies.

4.2 Materials and Methods

4.2.1 Study Participants

As discussed in chapter 2 (section 2.1),participants included in the current study were recruited from the Fisantekraal Community Clinic, in the outskirts of Cape Town, South Africa, as part of a previously reported large study; the African European tuberculosis Consortium (AE-TBC; www.ae-tbc.eu). As described previously, all study participants presented with signs and symptoms suggestive of TB disease, including persistent cough lasting ≥2 weeks and at least one of either fever, malaise, recent weight loss, night sweats, knowledge of close contact with a TB patient, haemoptysis, chest pain or loss of appetite, and were recruited prior to clinical or laboratory assessment for TB disease. Participants were eligible for the study if they were 18 years or older and willing to give written informed consent for participation in the study, including consent for HIV testing. Patients were excluded if they were pregnant, had not been residing in the study community for more than 3 months, were severely anaemic (haemoglobin <10 g/l), were on anti-TB treatment, had received anti-TB treatment in the previous 90 days or if they were on quinolone or aminoglycoside antibiotics during the past 60 days. Recruitment of study participants was done between November 2010 and November 2012. The study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch and written informed consent was obtained from all participants.

4.2.2 Sample Collection and Diagnostic Tests

As discussed in Chapter 2 (section 2.2.2), study participants fasted for at least one hour before saliva collection. Briefly, participants were asked to chew a sterile cotton swab (salivette) that was provided by the saliva collection kit manufacturer (Sarstedt, Numbrecht, Germany), for about 45 seconds. The swab was then removed from the participant's mouth with sterile forceps, inserted into a sterile tube provided by the manufacturer, and then transported to the laboratory at 4 - 8^{o}C. Upon arrival in the laboratory, the saliva samples were centrifuged at 1000g for 2 minutes and the supernatant harvested and stored at -80 ^{o}C until tested. After

microbiological confirmation of TB disease in study participants, sample collection was repeated for the culture confirmed TB patients at month 2 and month 6 after the initiation of TB treatment. As previously described in chapter 2 (section 2.3) and by Jacobs et al [83], sputum samples collected from all study participants were cultured using the MGIT method (BD Biosciences, Franklin Lakes, NJ, USA), after which positive MGIT cultures were examined for acid fast bacilli using the Ziehl-Neelsen technique (to check for contamination), followed by Capilia TB testing (TAUNS, Numazu, Japan), to confirm the isolation of organisms of the *M.tb* complex, before being designated as positive cultures.

4.2.3 Classification of Study Participants and Reference Standard

As previously described in chapter 2 (section 2.3) and by Jacobs et al [83], participants were classified as definite TB patients, probable TB patients, participants with other respiratory diseases (ORD) or questionable disease, using a combination of clinical, radiological, and laboratory findings. For the present discovery study however, only definite (culture positive) TB patients were included. Using an online tool (www.random.org), we then randomly selected individuals with ORD (n=33) for investigation alongside the definite TB patients with available saliva samples (n=18), due to the considerably higher numbers of individuals with ORD in our biobank. As described previously, individuals with ORD had a range of other diagnoses, including upper and lower respiratory tract infections (viral and bacterial infections, although attempts to identify organisms by bacterial or viral cultures were not made), and acute exacerbations of chronic obstructive pulmonary disease or asthma. Such investigations are not routinely done at primary health care settings, for example, where study participants were recruited.

4.2.4 Luminex Multiplex Immunoassay

The concentrations of 69 host markers, including markers that have not been widely investigated in the TB field (NCAM, transhyretin, MIP-4, antithrombin-III, GDF-15, ADAMTS13,

(in kits purchased from Merck Millipore, Billerica, MA, USA), and other host markers namely: alpha2 macroglobulin (A2M) , haptoglobin, C-reactive protein (CRP), serum amyloid P (SAP), procalcitonin (PCT), ferritin, tissue plasminogen activator (TPA), fibrinogen, serum amyloid A (SAA) (in kits purchased from Bio- Rad Laboratories, Hercules, CA, USA), vitronectin, extracellular matrix protein 1 (ECM1), vitamin D binding protein , sFas, granzyme A, sFasL, sCD137, granzyme_B, perforin, myoglobulin, P-selectin, lipocalin-2, thrombopoietin (TPO), stem cell factor (SCF), B-cell attracting chemokine 1 (BCA-1), epithelial neutrophil activating protein (ENA)-78, thymic stromal lymhpopoietin (TSLP), I-309(CCL-1), stromal cell derived factor 1 alpha (SDF-1α), IFN-γ, IFN-α 2, interferon inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1β, tumor necrosis factor (TNF)-α, TNF-β, vascular endothelial growth factor (VEGF), soluble CD40 ligand (sCD40L), apolipoprotein (Apo) A-1, Apo CIII, complement component 3, complement factor H (CFH), total plasminogen activator inhibitor 1 (PAI-1), brain-derived neurotrophic factor (BDNF), cathepsin D, myeloperoxidase (MPO), matrix metalloproteinase (MMP)-2, MMP-9, hemofiltrate CC chemokine 1 (HCC-1), α-1-antitrypsin, pigment epithelium derived factor (PEDF), complement C4, interleukin (IL)-17F, IL-17A, IL-22, IL-33, IL-21, IL-23, IL-25, IL-31, IL-28A, IL-16, IL-1β, IL-12(p40) and IL-13 (Merck Millipore, Billerica, MA, USA), were investigated in saliva samples from all the study participants. Experiments were performed in a blinded manner, according to the instructions of the kit manufacturers. All assays were read on the Bio-Plex platform (Bio-Rad), with the Bio-Plex Software version 6.1 used for bead acquisition and analysis.

4.2.5 Statistical Analysis

As discussed in chapter 2 (section 2.7),differences in the concentrations of host markers detected in saliva samples from TB patients and individuals with ORD were evaluated using the Mann-Whitney U test, for non-normally distributed data, whereas the student's t-test was used if data were normally distributed. The diagnostic abilities of individual host markers were investigated by receiver operator characteristics (ROC) curve analysis. The cut-off values for each analyte were determined by selecting the maximum values of Youden's index [102]. General discriminant analysis (GDA) was used to determine the predictive abilities of

combinations of markers for the diagnosis of TB disease, with leave one-out cross validation [103]. Differences in the expression profiles of host markers during the course of TB treatment were analysed by mixed model repeated measures analysis of variance (ANOVA), with Fisher's Least Significant Difference (LSD) post hoc testing. P-values ≤0.05 were considered significant. The data were analysed using Statistica (Statsoft, Ohio, USA) and Graphpad Prism version 5 (Graphpad Software Inc., CA, USA).

4.3 Results

A total of 51 study participants, 18 of whom were culture positive TB patients were investigated in this study. Twelve (23.5%) of the study participants were HIV infected. The clinical and demographic characteristics of study participants are shown in Table 4.1.

Table 4.1: Clinical and demographic characteristics of study participants. All the 18 TB patients included in the study were culture confirmed.

Number of participants	**All (n=51)**	**TB (n=18)**	**ORD (n=33)**
Male, n (%)	20 (39)	4 (22)	16 (48)
Mean age, (Years)±SD	36.4 ± 9.8	39.1 ± 9.7	35 ± 9.7
HIV infected, n (%)	12 (24)	4(22)	8 (24)
Quantiferon results			
Positive, n (%)	30 (60)	12 (71)	18 (55)
Negative, n (%)	19 (38)	4 (24)	15 (45)
Indeterminate, n (%)	1 (2)	1 (6)	0 (0)

TB= pulmonary tuberculosis, SD=standard deviation

4.3.1 Utility of Individual Salivary Host Markers in the Diagnosis of TB Disease

Of the 69 markers investigated in the current study, 23 (vitronectin, vitamin D binding protein, sFas, sFasL, sCD137, perforin, ADAMTS13, P-selectin, SCF, IL-28, Apo A-1, Apo CIII, CFH, NCAM, BDNF, MMP-2, MIP-4, complement C4, IL-17F, IL-25 and IL-23, IL-22, IL-33) were barely or not detectable in saliva samples and these markers were excluded from further analysis. When the baseline concentrations of the remaining 46 markers in the TB patients (n=18) were compared to the levels obtained in the 33 individuals with ORD with the Mann Whitney U or student's t test, only eight host markers were significantly different between the two groups. The median levels of IL-17A, IL-23 and the mean of concentrations of ECM-1 were significantly higher in the TB patients, whereas the median levels of A2M, SAP, IL-16, IL-1β, and the mean levels of granzyme B were significantly higher in the ORD group, whereas trends towards higher levels of myoglobulin and GDF-15 were observed in TB patients (Table 4. 2). When the diagnostic accuracies of individual host markers were investigated by ROC curve analysis, only two markers (IL-16 and IL-23) showed promise in the present study with area under the ROC curve (AUC) ≥0.70, whereas A2M, SAP, IL-17A and IL-1β performed with AUCs ≥ 0.67 (Table 4.2). Two additional markers (GDF-15 and IL-25) became significantly different between the TB patients and individuals with ORD, and/or showed diagnostic potential (AUCs of 0.72 and 0.53 respectively) only after data for HIV infected individuals were excluded. Representative plots showing the levels of some of the individual host markers with diagnostic potential, regardless of HIV infections status are shown in Figure 4.1.

Table 4.2: Median levels and interquartile ranges (in parenthesis) of host markers detected in baseline saliva samples from the TB patients (n=18) and individuals with ORD (n=33) and their diagnostic accuracies for TB disease. Only markers that showed significant differences or trends between groups with the Mann-Whitney U test are shown. The concentrations of A2M, SAP, ECM1, IL-23, myoglobulin and GDF-15 are in ng/ml. The concentrations of all the other markers are in pg/ml

Marker	**ORD**	**TB Disease**	**P value**	**AUC**	**Cut off value**	**Sensitivity % (95% CI)**	**Specificity % (95% CI)**
A2M	1966 (1546-3503)	1390 (1168-1926)	0.013	0.67 (0.5-0.84)	< 1351	50 (26-74)	88 (72-97)
ECM-1[a]	14.6±14.4	22.3±12.4	0.046	0.66 (0.49-0.84)	> 15.43	83 (59-96)	48 ((31-66)
GDF-15	0.02 (0.02-0.1)	0.05 (0.02-0.08)	0.088	0.64 (0.49-0.80)	> 0.0550	50 (26-74)	76 (58-89)
Granzyme B[a]	0.02±0.05	0.001±0.003	0.036	0.55	< 0.0150	100 (81-100)	27 (13-46)

IL-1β	36.4 (15.9-183.4)	16.9 (4.3-39.4)	0.027	0.69 (0.52-0.86)	< 7.285	39 (17-64)	97 (84-100)
IL-16	56.1 (22.0-144.3)	20.01 (0-59.39)	0.016	0.71 (0.55-0.87)	< 14.23	50 (26-74)	85 (68-95)
IL-17A	6.1 (2.2-10)	13.8 (3.9-29.4)	0.028	0.69 (0.53-0.85)	> 8.035	72 (47-90)	67 (48-82)
IL-23	0 (0-0)	0.3 (0-2.0)	0.0023	0.73 (0.59-0.88)	> 0.1550	61 (36-83)	85 (68-95)
Myoglobulin	0.4 (0.2-0.7)	0.6 (0.3-0.8)	0.097	0.64 (0.49-0.80)	> 0.2500	100 (81-100)	27 (13-46)
SAP	61.0 (5.5-61.0)	5.5 (5.5-5.5)	0.031	0.67 (0.51-0.82)	< 33.26	83 (59-96)	52 (34-69)

. [a]Mean ± standard deviation. Data for these host markers were normally distributed

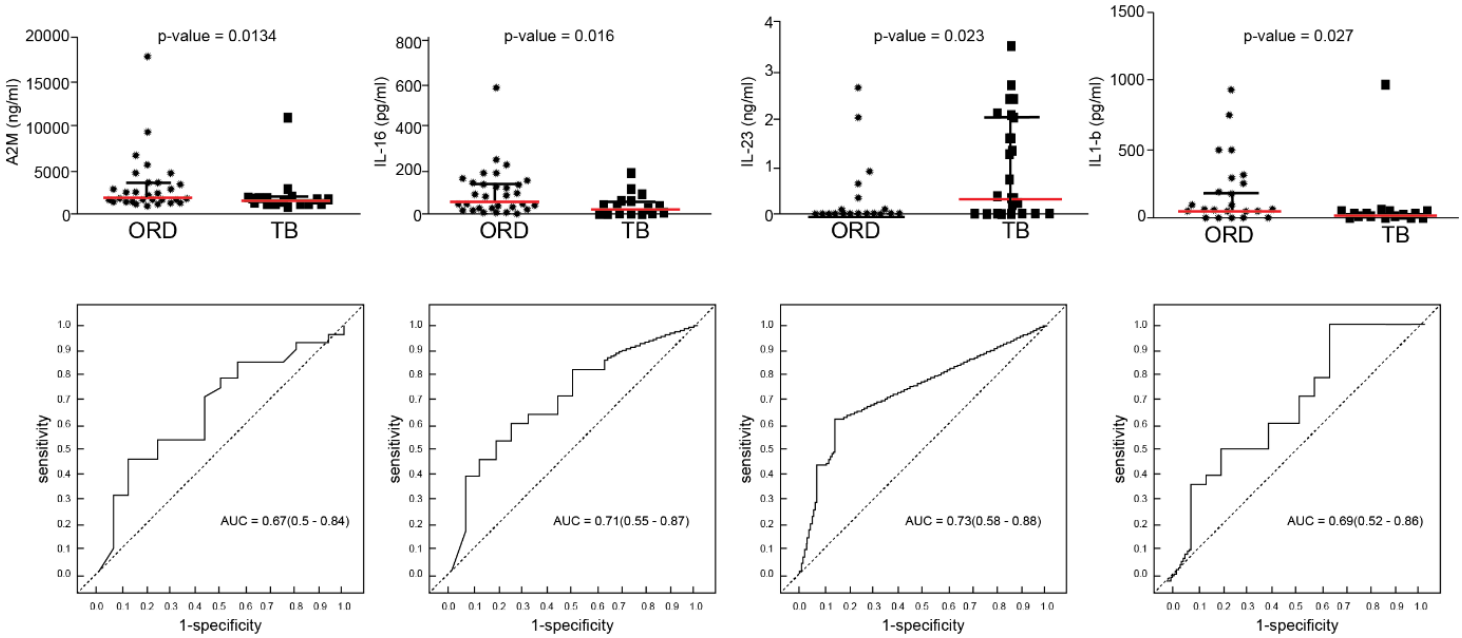

Figure 4.1: Scatter plots showing the concentrations of host markers detected in saliva samples from TB patients (n=18) and individuals with ORD (n=33) and receiver operator characteristics curves showing the accuracies of these markers in the diagnosis of TB disease. Representative plots are shown for A2M, IL-16, IL-23, and IL-17A. Error bars in the scatter dot plots represent the median with interquartile range.

4.3.2 Utility of Multi-Saliva Marker Combinations in the Diagnosis of TB Disease

When the data obtained from all study participants were fitted into General Discriminant Analysis (GDA) models, regardless of HIV status, combinations between up to five different host markers showed potential in the diagnosis of TB disease. A three-marker biosignature comprising of granzyme B, fibrinogen and A2M diagnosed TB disease with a sensitivity of 94.4% (95% CI, 74.0-99.9%) and specificity of 62.1% (95% CI, 40.6-78.5%) in the resubstitution classification matrix and sensitivity of 94.4% (95% CI,72.7-99.9%) and specificity of 58.6% (95% CI, 38.9-76.5%) after leave-one-out cross validation. However, the most optimal diagnostic biosignature irrespective of HIV status was a combination between five markers

(IL-1β, IL-23, ECM-1, HCC1 and fibrinogen) which diagnosed TB disease with a sensitivity 88.9% (95% CI,76.7-99.9%) and specificity of 89.7% (95% CI, 60.4-96.6%) in the resubstitution classification matrix, and with the same accuracy (sensitivity of 88.9% and specificity of 89.7%) after leave-one-out cross validation. The positive and negative predictive values of the five-marker biosignature were 89.7% (95% CI, 76.7-97.8%) and 88.9% (95% CI, 65.3-98.6%) respectively.

When the GDA procedure was repeated after excluding the HIV infected individuals, two eight-marker biosignatures diagnosed TB disease with high accuracies. A biosignature comprising of granzyme A, GDF-15, SAA, IL-21, ENA-78, IL-12(p40), IL-13 and PAI-1,diagnosed TB disease with sensitivity of 93% (95% CI, 77.2-99.9%) and specificity of 100% (95% CI, 75.3-100%) in the resubstitution classification matrix, and after leave-one-out cross validation, whereas a biosignature comprising of ECM1, myoglobulin, HCC1, IL-21, ENA-78, TPA, IL-12(p40) and IL-13 diagnosed TB disease with sensitivity of 100% (95% CI, 83.2-100%) and specificity of 95% (95% CI, 68.1-99.9%) in the resubstitution classification matrix, and after leave-one-out cross validation. IL-1β was the most frequent analyte in biosignatures, appearing in 93% of the biosignatures generated for diagnosing TB disease regardless of HIV infection status (Figure 4.2).

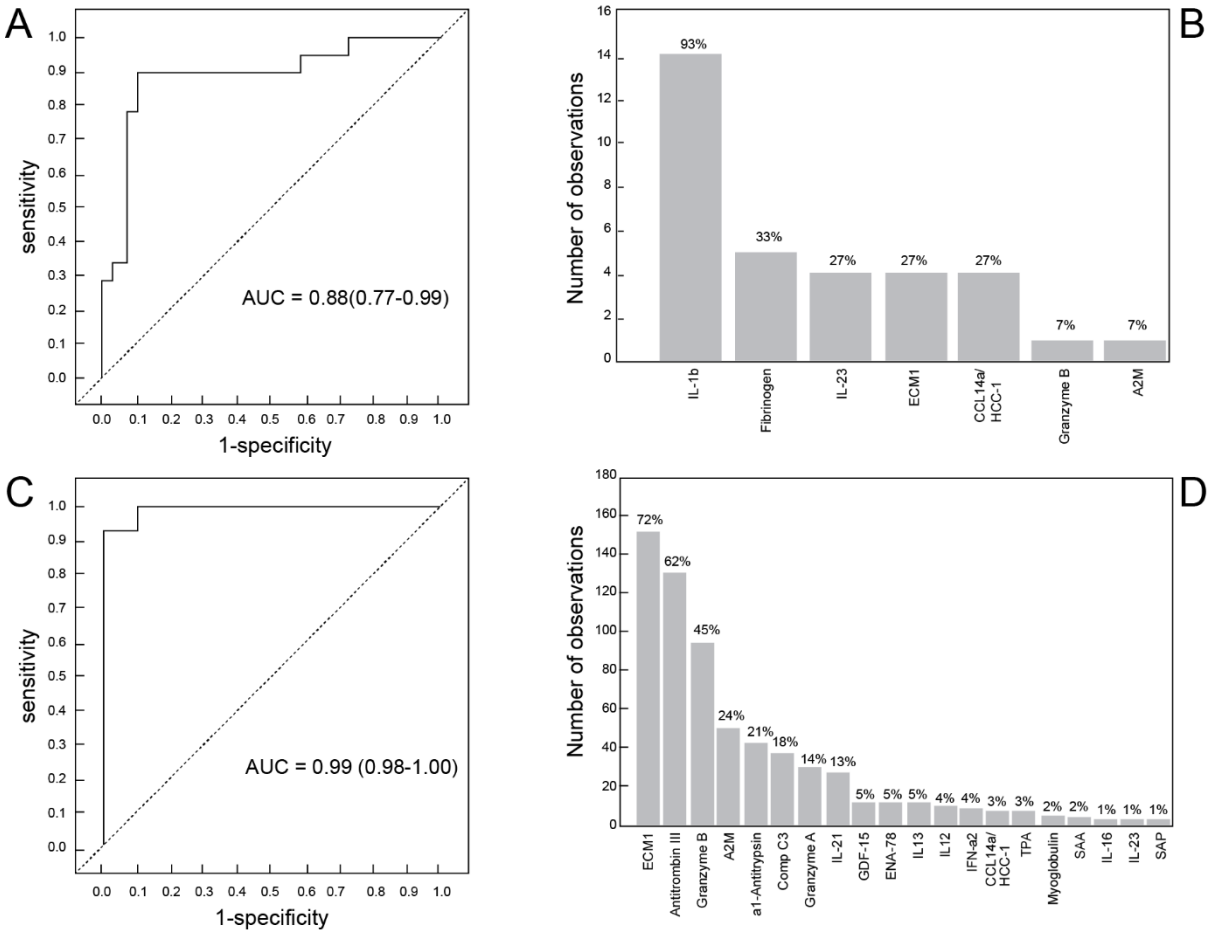

Figure 4.2: Accuracy of salivary multi-marker models in the diagnosis of TB disease. Receiver operator characteristics (ROC) curve showing the accuracy of the optimal five-marker biosignature (IL-1β, IL-23, ECM1, HCC1, fibrinogen) in the diagnosis of TB disease in all study participants, regardless of HIV infection status (A), frequency of analytes in top general discriminant analysis (GDA) models that most accurately classified individuals as TB patients or ORD irrespective of HIV status (B), ROC curve showing the accuracy of the most accurate eight-marker biosignature (granzyme A+PAI-1+GDF-15+SAA+IL-21+ENA-78+IL-12(p40) and IL-13) in the diagnosis of TB disease after exclusion of HIV positive individuals (C), and frequency of analytes in the top GDA models that most accurately classified study participants as TB or ORD in the absence of HIV infection (D). The bar graphs (B and D) indicate the frequency of analytes in the most accurate GDA models.

4.3.3 Changes in the Concentrations of Host Biomarkers during the Course of TB Treatment

To investigate whether any of the 46 detectable host markers could potentially be used as markers to monitor TB treatment response, we evaluated the concentrations of the markers in saliva samples that were collected from TB patients at baseline, and at months 2 and 6, following the start of TB treatment. Of the 18 TB patients included in the study, all 18 (100%) and 13 (72%) returned to the clinic and provided samples at months 2 and 6 respectively, and these samples were used in this part of the study. The salivary concentrations of 8 host markers changed significantly during the course of treatment. There was a significant decrease in the levels of haptoglobin and CRP from baseline to month 2, but a significant increase in ENA-78 levels over the same period (Figure 4. 3, Table 4.3). The levels of ENA-78 continued to increase towards month 6. The levels of IP-10, MIP-1β and VEGF increased significantly when baseline levels were compared to end of treatment (month 6) levels, whereas the levels of lipocalin-2 significantly decreased and a trend towards decreasing levels of ECM1 from baseline to month 6 was observed (Figure 4.3, Table 4.3). There were no significant differences in transthyretin levels between diagnosis and month 2, but the increase in the concentrations of the protein became significant by the end of treatment (Figure 4.3, Table 4.3).

Table 4.3: Mean values of host makers detected in saliva samples of TB patients at baseline, month 2 and month 6 (after the start of TB treatment). Data were analysed using mixed model repeated measures analysis of variance, with Fisher's Least Significant Difference post hoc testing. The mean values shown (95% confidence intervals in brackets) are the least squared means. Significant P-values are in bold.

	Time point			**P value**		
Marker	Baseline	Month 2	Month 6	Baseline Vs. M2	M2 Vs. M6	Baseline Vs. M6
CRP	16 (12.7-19.5)	13.2 (9.8-16.6)	14.2 (9.9-18.5)	**0.015**	0.27	0.21
ECM-1	22 (15.9-28.2)	15.5 (8.4-22.6)	12.6 (4.4-20.7)	0.12	0.63	0.063
ENA -78	35 (8.6-61.6)	107.7 (42.2-173.1)	125 (39-211.4)	**0.015**	0.88	**0.020**
Haptoglobin	1664.6 (905.1-2424.1)	845 (295.2-1398.7)	935.2 (181.91-688.4)	**0.029**	0.81	0.076
IL-17A	13.4 (9-17.9)	8.2 (4.7-11.7)	9 (3.6-14.3)	0.064	0.74	0.16
IL-23	0.81 (0.36-1.25)	0.38 (0.036-0.72)	0.35 (-0.1-0.81)	0.083	0.83	0.17

IP-10	70 (24.3-115.4)	113.4 (67.7-159.1)	137.6 (59.3-216)	0.11	0.49	**0.040**
Lipocalin-2	1415,5 (750.2-2080.8)	1393,2 (749.3-2037.1)	1097.6 (425.58-1769.646)	0.62	0.053	**0.027**
MIP-1 β	16.2 (10.1-22.3)	17.6 (10-25.3)	23.7 (11.3-36)	0.38	0.098	**0.022**
SDF-1a	421,2 (324.4-518)	275.4 (151.7-399)	333.5 (164-503.1)	0.080	0.51	0.33
Transthyretin	1810,9 (1577.4-2044.5)	1817.6 (1611.4-2023.9)	1922.2 (1686.4-2158)	0.75	**0.047**	0.12
VEGF	355.5 (261.4-450)	392.2 (304-480.4)	447 (359-535)	0.18	0.30	**0.034**

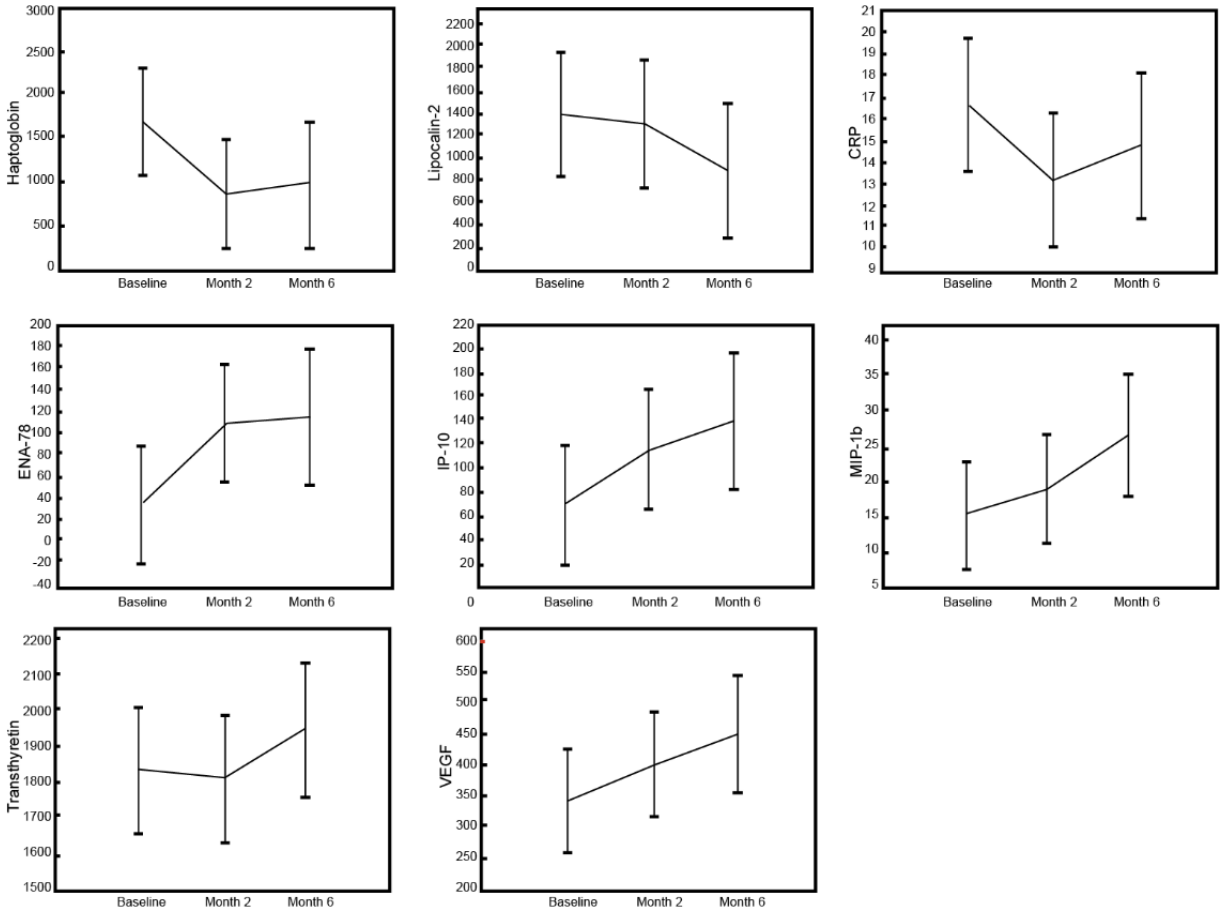

Figure 4.3: Changes in the concentrations of host markers in saliva samples from TB patients undergoing TB treatment. Saliva samples were collected from all study participants at baseline (before the start of treatment) and at months 2 and 6 after initiation of TB treatment. Error bars indicate the Least Squared means with 95% Confidence Intervals.

4.3.4 Comparison of the Levels of Host Markers Detected in Saliva to the Levels Obtained in Plasma

As the study participants whose samples were evaluated in this chapter were also evaluated in chapter 3, we compared the levels of all the 69 host markers evaluated in saliva samples in this chapter to the levels obtained in plasma samples from the same study participants in chapter 3. This was necessary given that 23 of the 69 host markers evaluated were barely detectable in saliva samples as mentioned earlier in this chapter. Data on differential expression of the host markers in the two sample types may be beneficial when designing future experiments. Using data obtained from the manufacturer's package inserts, we

evaluated the proportion of patients in whom the concentrations of the different host markers in saliva and plasma were ≥ the published minimum detectable concentration (MDC). P-values for differential expression of the markers in saliva and plasma samples were computed using the Mann Whitney U test.

Out of the 69 markers evaluated, information about the MDC was not available for 9 (kits purchased from Bio Rad laboratories) namely; A2M, haptoglobin, CRP, SAP, PCT, ferritin, TPA, fibrinogen and SAA. The concentrations of three of the proteins (lipocalin-2, Cathepsin D and TNF-α) were higher than the MDC +2 standard deviations in both sample types, in all study participants whereas values for none of the proteins in saliva and 14 proteins in plasma were above the MDC in all samples respectively (see table 4.4).

The mean and/or median concentrations of eight of the proteins (lipocalin-2, TSLP, IL-16, IL-1β, IFN-α2, IL-13, VEGF and IL-17A) were significantly higher in saliva samples when compared to plasma, and the levels of seven (granzyme A, granzyme B, IL-28, IFN-γ, TNF-β, MPO and IL-21) showed no difference in expression levels between saliva and plasma, whereas all the remaining markers were significantly higher in plasma than in saliva (Table 4.4).

Table 4.4: Proportion of study participants with host markers above the minimum detectable concentration in saliva and plasma and differences in median levels detected in the two sample types. The MDC values shown include 2x standard deviations (MDC +2SD) whereas values in parenthesis are the interquartile ranges.

Marker	**MDC+ 2SD (pg/ml)**	**% > MDC + 2SD**	**Saliva Median (IQR)**	**% > MDC+ 2SD**	**Plasma Median (IQR)**	***P* value**
Host markers more abundantly expressed in saliva						
#Lipocalin-2	0.004	100	1013 (524.0- 1838)	100.0	534.4 (306.4- 830.9)	0.0005
TSLP	3.1	8.5	0.060 (0.0- 0.88)	10.6	0.0 (0.0-0.0)	0.0005
IL-16	9.1	78.7	43.27 (12.35- 109.4)	29.8	0.0 (0.0-14.1)	<0.0001
IL-1β	1.0	87.2	31.76 (9.75- 53.93)	70.0	2.37 (0.31- 12.86)	<0.0001
IFN-α2	4.8	95.7	191.4 (85.64- 377.0)	70.2	38.18 (0.0- 219.0)	0.0009
IL-13	1.9	76.6	7.44 (2.83- 16.92)	27.7	0.0 (0.0-6.06)	<0.0001
VEGF	47.9	95.7	458.6 (219.5- 656.4)	63.8	200.6 (53.88- 601.3)	0.0118
IL-17A	2.8	80.85	8.43 (2.80- 17.04)	34.0	0.0 (0.0-14.26)	0.0006

Host markers more abundantly expressed in plasma						
#A2M	NA	NA	1645 (1309-2543)	NA	1258000 (979700-1632000)	<0.0001
#Haptoglobin	NA	NA	772.2 (86.17-3152)	NA	9943000 (792200-36000000)	<0.0001
#CRP	NA	NA	17.85 (13.59-22.74)	NA	4884 (1722-27610)	<0.0001
#SAP	NA	NA	5.490 (5.49-61.02)	NA	23820 (18280-30150)	<0.0001
PCT	NA	NA	1607 (891.2-2613)	NA	8336 (7520-9520)	<0.0001
Ferritin	NA	NA	3270 (1573-5581)	NA	103300 (54920-177600)	<0.0001
TPA	NA	NA	397.5 (139.3- 749.5)	NA	6624 (5825- 7562)	<0.0001
Fibrinogen	NA	NA	1943 (1303- 2811)	NA	36000 (36000- 36000)	<0.0001
#SAA	NA	NA	0.0 (0.0- 35.29)	NA	8347	<0.0001

					(2002- 30730)	
#Vitronectin	0.021	2.1	0.0 (0.0-0.0)	97.9	289000 (244300- 322700)	-
#ECM-1	0.003	89.4	19.71 (2.320- 19.71)	97.9	8875 (6755- 11960)	<0.0001
#Antithrombin III	0.209	89.4	1739 (1558- 1923)	97.9	659100 (509800- 803100)	<0.0001
#Vitamin D Binding Protein	0.20	2.1	0.0 (0.0-0.0)	97.9	437600 (379800- 518800)	<0.0001
#sFAS	0.2261	6.4	0.0 (0.0-0.0)	83.0	6.36 (3.3- 10.52)	<0.0001
#sFASL	0.0089	8.5	0.0 (0.0-0.0)	95.7	0.02 (0.01- 0.02)	<0.0001
#sCD137	0.0017	6.4	0.0 (0.0-0.0)	91.5	0.01 (0.01- 0.02)	<0.0001
#Perforin	0.0075	6.4	0.0 (0.0-0.0)	97.9	4.63 (3.48-6.38)	<0.0001
#Myoglobin	0.007	91.5	0.53 (0.3-0.79)	97.9	39.24 (29.33-55.55)	<0.0001

#ADAMTS-13	0.418	2.1	0.0 (0.0-0.0)	100	3359 (2561-6291)	<0.0001
#P-selectin	0.119	0.0	0.0 (0.0-0.0)	100	299.9 (174.4-591.1)	-
#GDF-15	0.0006	97.9	0.04 (0.02-0.07)	100	29.94 (15.97-59.62)	<0.0001
TPO	37.9	10.6	0.0 (0.0-2.21)	29.8	15.42 (0.052-61.69)	<0.0001
*SCF	5.6	0.0	0.34 (±1.09)	10.6	4.17 (±14.64)	0.0061
BCA-1	1.3	29.8	0.45 (0.0-1.85)	97.9	54.17 (32.39-132.8)	<0.0001
ENA-78	7.2	31.9	28.63 (0.0-146.8)	97.9	226.8 (134.1-433.4)	<0.0001
I-309	1.4	2.1	0.34 (0.0-0.72)	46.8	1.42 (1.09-2.36)	<0.0001
SDF-1α	55.8	72.3	445.4 (0.0-624.6)	85.1	784.1 (391.1-1329)	<0.0001
IP-10	14.0	95.7	38.12 (20.85-138.4)	100	828.3 (498.2-2471)	<0.0001
MIP-1β	4.8	78.7	13.55 (4.85-29.82)	97.9	54.75 (21.12-133.6)	<0.0001

TNF-α	1.1	100	7.97 (3.26-13.89)	100	14.11 (7.36-24.26)	0.0038
sCD40L	9.9	14.9	3.29 (1.26-5.43)	97.9	391.3 (253.8-532.4)	<0.0001
#APO-A1	0.396	0.0	0.0 (0.0-0.0)	97.9	362300 (254900-503900)	-
#APO-CIII	0.002	6.4	0.0 (0.0-0.0)	100	454500 (327400-594400)	<0.0001
#Complemet C3	0.025	36.2	0.0 (0.0-314.7)	97.9	54610 (36250-78690)	<0.0001
#Transthyretin	0.029	87.2	1860 (1760-2059)	100	401300 (263800-556800)	<0.0001
#Complement FH	0.075	0.0	0.0 (0.0-0.0)	100	748900 (563500-899300)	-
PAI-1	0.92	39.3	0.0 (0.0-372.6)	97.9	103100 (74110-142600)	<0.0001
NCAM	13.48	0.0	0.0 (0.0-0.0)	100	422900 (346400-575000)	-

BDNF	0.47	2.1	0.0 (0.0-0.0)	91.5	4291 (3156-7814)	<0.0001
Cathepsin D	23.91	100	202800 (125600-321400)	100	563800 (305900-894500)	<0.0001
MMP-2	260	17.0	0.0 (0.0-0.0)	97.9	585800 (471100-686700)	<0.0001
MMP-9	4.4	97.9	234200 (79950-375600)	100	491000 (325400-706200)	<0.0001
HCC-1	2.0	91.5	394.2 (337.3-462.6)	100	119400 (85560-149500)	<0.0001
#α1-antitrypsin	0.085	93.6	515.5 (384.4-567.8)	100	8207 (5478-36950)	<0.0001
#PEDF	0.016	78.7	25.25 (9.68-48.46)	93.6	11910 (9422-14050)	<0.0001
#MIP-4	0.0049	14.9	0.0 (0.0-0.0)	100	95.09 (45.77-191.2)	<0.0001
#Complement C4	0.129	0.0	0.0 (0.0-0.0)	87.2	126600 (63440-185900)	-
#*IL-17F	0.016	0.0	0.0	31.9	0.014	-

			(±0.0)		(±0.023)	
#IL-22	0.032		0.0 (0.0-0.0)	93.6	0.28 (0.16-0.65)	<0.0001
IL-33	10.9	0.0	0.0 (0.0-0.0)	83.0	129.5 (45.96-233.4)	-
#IL-23	0.169	34.0	0.0 (0.0-0.75)	89.4	3.52 (1.6-7.91)	<0.0001
#IL-25	0.186	10.6	0.0 (0.0-0.0)	72.3	1.59 (0.75-3.44)	<0.0001
#IL-31	0.041	4.3	0.0 (0.0-0.0)	85.1	0.28 (0.11-0.76)	<0.0001
<u>No differences in the levels expressed in saliva and plasma</u>						
#Granzyme A	0.0257	25.5	0.0 (0.0-0.05)	21.3	0.0 (0.0-0.02)	0.49
#*Granzyme B	0.0011	19.1	0.019 (0.078)	14.9	0.0026 (±0.0092)	0.47
*IL-28	7.9	2.1	3.91 (±0.99)	17.0	20.07 (±67.69)	0.25
IFN-γ	1.1	87.2	5.63 (2.18-16.58)	83.0	9.81 (2.57-48.74)	0.2
*TNF-β	1.9	23.4	1.74	23.4	10.45	0.53

			(±4.16)		(±32.61)	
MPO	550	97.9	2697000 (1150000-7500000)	100	2703000 (1352000-4760000)	0.61
IL-21	3.3	87.2	20.02 (9.7-41.56)	91.5	23.93 (11.66-65.99)	0.32

*Mean ± standard deviation are shown for host markers whose values were normally distributed. # Marker levels are expressed in ng/ml. All other analyte levels are in pg/ml.

4.4 Discussion

In the present study, we evaluated the potential usefulness of 69 host markers as candidates for the salivary immunological diagnosis of TB disease and monitoring of the response to TB treatment. Although significant differences were observed in the concentrations of some of the markers including A2M, SAP, IL-17A, IL-1β, ECM1, IL-16, IL-23 and granzyme B , our study confirmed the notion that optimal diagnosis of TB disease may only be possible using biosignatures containing multiple analytes, as opposed to single markers. The most optimal biosignature identified in the present study was a five-marker signature comprising of IL-1β, IL-23, ECM-1, HCC1 and fibrinogen, which diagnosed TB disease with a sensitivity of 88.9% and specificity of 89.7%, regardless of HIV infection status. After excluding the HIV positive individuals, eight-marker biosignatures diagnosed TB disease with both sensitivity and specificity up to 100%, depending on the markers in the biosignature.

TB immunological tests may be more beneficial especially in resource-constrained settings, as they may be easily converted into point-of-care screening tests. Such tests would yield the highest impact if based on host markers that are detectable in easily available samples such as saliva, and are preferably based on the lateral flow technology. Out of the 69 host markers investigated in the current study, fibrinogen, antithrombin III, A2M, SAA, SAP, IL-1β, IL-23, IL-21, IL-13, IL-12(p40), HCC1, ENA-78, GDF-15, myoglobulin, TPA, granzyme A, granzyme B, PAI-1, α-1-antitrypsin, and complement C3, featured in diagnostic biosignatures for TB disease,

thereby indicating that they may be candidate biomarkers for inclusion into future salivary diagnostic validation studies. Although most of these proteins are well-known and have been investigated widely in other diseases including TB [120, 140–142], most of them have not previously been investigated in saliva samples, especially in the context of active TB disease.

In a previous small study comparing the expression of host markers in saliva to serum, vast differences were observed in the expression of host markers in saliva and serum, with some salivary host markers showing potential as diagnostic candidates [84]. In a relatively larger follow-up study [83], some of the potential host markers identified in [84] continued to show promise, with new host markers, and also markers showing potential as TB treatment response candidates identified [83]. IL-1β and IL-13 were amongst the markers that were more abundantly expressed in saliva, and IL-1β was included in top salivary biosignatures for the diagnosis of TB disease [84], as also observed in the current study. In addition to these two cytokines, other markers that showed potential in the present study as determined by p-values for differences between TB and ORD or inclusion into top biosignatures, and which were also identified in the two previous saliva based studies [84,83] as diagnostic candidates including fibrinogen, A2M, CRP, IP-10, MIP-1β, IL-17, VEGF and SAP, may be strong candidates for further investigation in future larger studies. Despite the agreement observed between the current and the two previous saliva based studies [84,83], we observed some differences between the current, and these previous studies. Although A2M and SAP were identified as important diagnostic candidates in this and the previous studies [84,83], we observed higher levels of A2M and SAP in saliva samples from individuals with ORD in the present study, in comparison to higher levels of these markers in TB patients in one of the relatively larger previous studies [83]. Although it may not be possible to completely explain the reasons for these differences, the use of limited numbers of study participants and the real potential for false discoveries exist, as patients used in these studies were not homogenous. The study by Phalane et al [84] as a small case-control study which was primarily aimed at investigating whether there were any differences in the expression of host markers between saliva and serum samples. The previous study by Jacobs et al [83] was a relatively larger study that was conducted on individuals that presented with signs and symptoms of TB, who were consecutively recruited, much like the study participants included in the current study. However only culture positive TB patients (n=18), four of whom were HIV infected and

randomly selected individuals with ORD were included in the current study, due to high costs associated with investigating the relatively large numbers of host markers in this study. Although the findings from all these studies require careful interpretation because of the heterogeneity in the studies, the potential host markers so far identified through these studies may be regarded as candidate biomarkers, requiring validation in larger studies. We also identified new salivary diagnostic candidate markers in the current study including IL-16, IL-23 and ECM-1.

IL-16 is a pro-inflammatory cytokine and a ligand for $CD4^+$ T lymphocytes [143] and in the context of HIV, has been shown to inhibit the replication of the virus, with serum levels decreasing with HIV disease progression [144]. IL-23 is important in the Th17 pathway in *M.tb* infection, and is generally produced by alveolar macrophages and dendritic cells [144]. ECM-1 is a glycoprotein and plays a role in angiogenesis, and has been implicated in tumor progression [145,146]. IL-23 was amongst the markers included in the optimal five-marker signature identified in the present study and also showed promise individually as a diagnostic candidate for TB disease. Although not expressed at high levels, the concentrations of IL-23 and ECM1 were significantly higher in TB patients, with IL-16 levels higher in patients with ORD in the current study. These markers warrant inclusion into analyte panels in future validation studies.

We also investigated salivary levels of newly identified serum and plasma diagnostic host markers including NCAM, MIP-4 [147], transthyretin, Apo A-1 amongst others [82]. The levels of Apo A-1, NCAM and MIP-4 were not detectable in saliva samples in the current study, whereas transthyretin levels were not significantly different between TB patients and individuals with ORD, even though transthyretin showed potential as a marker of TB treatment response. Although salivary GDF-15 levels showed a trend towards higher levels in TB patients, the data obtained in the current study indicates that most of these markers may not be useful as diagnostic candidates in saliva, and require further investigation.

In addition to being potential salivary TB diagnostic candidates, Il-17A, IL-23 and ECM-1 also showed potential as markers for TB treatment response. The high concentrations observed for these markers in TB patients in comparison to ORD at baseline, reduced (although not always significantly) in the course of TB treatment. Our observations for CRP, IP-10 and VEGF as salivary candidate markers for monitoring of the response to treatment are in agreement with previous investigations [83,132,148]. As discussed previously [83], larger studies are required to validate the potential of these markers as biomarkers for TB treatment response. Such studies should include samples collected at earlier time points, for investigation of markers of early TB treatment response, and should also investigate whether these markers are informative in the prediction of month 2 culture outcome, favourable treatment outcome (clinical cure) and poor outcomes (relapse and treatment failure).

When comparing the levels of 69 markers in saliva samples to the levels obtained in plasma for the same study participants, differences in the expression of host markers were found between the two sample types. Eight markers (Lipocalin-2, TSLP, IL-16, IL-1β, IFN-α2, IL-13, VEGF and IL-17A) were found to be significantly higher in saliva samples. Our findings are in agreement to a previous study conducted in our laboratory which also showed significantly higher levels of expression for IL-1β, IL-13, VEGF and IL-17A in saliva [84]. Both IL-13 and IL-1β were included in biosignatures generated in the present study and therefore further supports the idea of saliva being a valuable sample type for research in TB biomarker discovery.

The main limitation of our study was the small sample size. As this was largely a discovery study, this might not be a major problem especially as we also confirmed findings from previous studies [84,83]. The few discrepancies between the markers identified in this and previous studies [84,83], strengthen the argument for the need for further validation of these candidate markers in larger studies before they may be considered for incorporated into point-of-care lateral flow test platforms. Although incorporation of host markers identified in the laboratory using platforms such as the Luminex technology into point-of-care tests is challenging, recent advances in technology have made such tasks possible. A prototype of

such a point-of-care test was recently developed and successfully investigated in a recent Pan-African study [80], with further development of the platform ongoing (www.screen-tb.eu). The strength of such a test, based on validated biomarkers including the ones identified in the present study include non-invasive sampling, and such a test might be very useful in the diagnosis of paucibacillary TB. The effect of HIV infection and other comorbidities such as diabetes mellitus on salivary biomarkers requires investigated in future studies. Furthermore, such studies should also include individuals with confirmed other diseases that present with symptoms that are similar to TB including pneumonia.

In conclusion, we have identified candidate salivary biosignatures with potential in the diagnosis of TB disease and monitoring of TB treatment response. Our findings require further validation in larger studies, before being considered as candidate markers for point-of-care diagnostic tests.

Chapter 5

Utility of antibodies against recently identified *Mycobacterium tuberculosis* proteins as candidates for the diagnosis of TB disease and monitoring of treatment response

5.1 Introduction

Tuberculosis (TB) remains one of the leading death causing infectious diseases worldwide, being responsible for nearly 1.5 million deaths in 2014 [9]. The control of the disease largely depends on early case detection and proper treatment, therefore in order to reduce the burden of the disease, rapid and accurate tools for both the diagnosis and monitoring of TB treatment response are required. Existing methods for the diagnosis of TB disease still largely rely on initial clinical suspicion and subsequent microbiological confirmation by Ziehl-Neelsen staining and culture. However these methods have several drawbacks: -smear microscopy is the most common and affordable method used especially in resource poor settings, however it's sensitivity is compromised and in addition this approach is also unable to distinguish between live and dead bacilli, making it impractical to be used as a tool to monitor TB treatment response [59, 99]. Although the gold standard test for TB (culture) is more sensitive, this method requires bacterial growth of up to 6-8 weeks before case detection and is consequently also not suitable for monitoring TB treatment response. The automated gene amplification test GeneXpert (Cepheid Inc., Sunnyvale, USA) is more rapid and is also able to detect resistance to rifampicin [63]. However the use of the test is limited in resource constrained settings due to high operating costs and infrastructural needs. Immunodiagnostic assays may be useful for both the diagnosis of TB disease and monitoring of the response to treatment especially since they have the potential to be easily adaptable into rapid, point-of-care tests, which would be suitable at primary health care centres in resource-constrained settings.

Previous studies have shown that exposure to *M.tb* elicits antibody production to various antigens [149, 150]. TB serological diagnostic assays have been widely investigated, and have been improved over the years by using highly purified recombinant antigens, as previous generations of the assays were criticized for their low sensitivity in TB endemic regions [151,79]. As demonstrated in previous chapters of this thesis and in several previous studies, inflammatory biomarkers may be very useful in the diagnosis of TB disease [82, 98, 100]. As these inflammatory biomarkers are known to not be very disease-specific, consequently having sub-optimal specificity for TB, it is not known whether combining the cytokine biomarkers (traditionally good sensitivity but poor specificity) with antibodies which are traditionally very specific (but with poor sensitivity) will result in a better diagnostic biosignature for TB disease. A biosignature making use of both classes of biomarkers may therefore benefit from the strengths of each individual diagnostic approach.

Previous studies conducted in our laboratory evaluated antibodies against novel *M.tb* antigens for their ability to diagnose TB disease and as tools for monitoring of the response to TB treatment. These studies revealed that a combination of multiple antibody classes (Ig A, Ig G, Ig M) against multiple *M.tb* antigens including LAM, Tpx and PPE proteins ascertained TB disease with high accuracy [78]. Furthermore, combinations between other antibodies including anti-alanine dehydrogenase IgG, anti-Tpx IgG, anti-ESAT-6 IgG and anti-ESAT-6 IgA, measured prior to TB treatment initiation showed promise as markers for the prediction of early TB treatment response [152]. However, in other work done by Legesse et al, it was demonstrated that the discriminatory ability of *M.tb*-specific IgA antibodies is better than IgG antibodies between active TB and endemic controls in Africa [153]. This was also the case between healthy individuals with close contact to a TB patient and individuals with no contact with such patients in another study [154], with other studies also demonstrating a protective role for IgA in murine models of mycobacterial infection [155]. Contrary to previous work that was conducted in our laboratory, the present study focused on IgA antibodies. Furthermore, we explored the utility of IgM antibodies against LAM as this kit was not available in previous panels evaluated in our high endemic TB setting.

The aim of the present study was therefore to explore the diagnostic potential of Ig A antibodies against six *M.tb* specific antigens, and IgM antibodies against LAM in plasma samples from active TB cases and individuals with other respiratory diseases (ORD). To investigate whether the diagnostic accuracy for TB disease would improve if anti-*M.tb* antibodies were used in combination with cytokine biomarkers, we evaluated a diagnostic approach in which the two classes of biomarkers were combined. Furthermore, we also evaluated the potential of the antibodies as markers for monitoring of TB treatment response.

5.2 Materials and Methods

5.2.1 Study Participants

As discussed in chapter 2 (section 2.1) , participants enrolled into the present study were individuals who presented with signs and symptoms requiring investigation for TB disease at the Fisantekraal Community Clinic in the outskirts of Cape Town, South Africa. The study was a sub-study of a larger diagnostic biomarker project (the African European Tuberculosis Consortium), that was ongoing at the study site and at field sites situated in six other African countries (www.ae-tbc.eu).

5.2.2 Samples and Laboratory Experiments

Plasma samples were collected and processed as described in chapter 2 (section 2.2.1). ELISA experiments were performed on all study participants as described in chapter 2 (section 2.6), following the optimization experiments reported in section 2.8.3.Data were analysed using Statistica (Statsoft, Ohio, USA) and Graphpad Prism version 5 (Graphpad Software Inc., CA, USA) as described in chapter 2 (section 2.7) . Briefly, differences in the antibody responses between TB patients and individuals with ORD were analysed using the Mann-Whitney U test. The diagnostic abilities of individual antibody responses were assessed by receiver operator characteristics (ROC) curve analysis. The cut-off values for each antibody and associated sensitivity and specificity were determined by selecting the maximum values of Youden's index [102]. The predictive abilities of combinations of between different antibodies and

between antibodies, cytokines and other parameters were investigated by general discriminant analysis (GDA), with leave-one-out cross validation [103]. Differences in the levels of antibodies during the course of TB treatment were analysed using mixed model repeated measures analysis of variance (ANOVA), with Fisher's Least Significant Difference (LSD) post hoc testing. P-values ≤0.05 were considered significant.

5.3 Results

A total of 156 study participants, 22 of whom were definite and 4 that were probable TB patients (please see table 2.1) were investigated in this study. The mean age of all study participants was 37 ± 11.2 years and 28 (18%) were HIV infected. The clinical and demographic characteristics of study participants enrolled for this chapter are shown in table 5. 1.

Table 5. 1: Clinical and demographic characteristics of study participants.

Number of participants	**All Participants (n=156)**	**Definite TB (n=22)**	**Probable TB (n=4)**	**All TB (n=26)**	**ORD (n=130)**
Males, n (%)	65 (42)	6 (27)	0 (0)	6 (23)	59 (45)
Mean age, (Years)±SD	37 ± 11.2	39 ±9.9	40 ± 11.4	39 ± 9.9	37 ±11.5
HIV Infected, n (%)	28 (18)	4 (18)	0 (0)	4 (15)	24 (18)
Quantiferon results					
Positive, n (%)	104 (70)	16 (80)	3 (75)	19 (79)	85 (69)
Negative, n (%)	43 (29)	3 (15)	1 (25)	4 (17)	39 (31)
Indeterminate, n (%)	1 (1)	1 (5)	0 (0)	1 (4)	0 (0)
Symptoms at enrolment					
Cough, n (%)	156 (100)	22 (100)	4 (100)	26 (100)	130 (100)
Weight loss, n (%)	103 (66)	16 (23)	4 (100)	20 (77)	83 (64)
Sputum production, n (%)	155 (99)	22 (100)	4 (100)	26 (100)	129 (99)
Night sweats, n (%)	110 (71)	19 (86)	4 (100)	23 (88)	87 (67)
Malaise, n (%)	87 (56)	15 (68)	3 (75)	18 (69)	69 (53)
Fever, n (%)	44 (28)	10 (45)	2 (50)	12 (46)	32 (25)
Anorexia, n (%)	80 (51)	13 (59)	4 (100)	17 (65)	63 (48)
Haemoptysis, n (%)	23 (15)	4 (18)	1 (25)	5 (19)	18 (14)
Chest pain, n (%)	115 (74)	19 (86)	3 (75)	22 (85)	93 (72)
Short Breath, n (%)	129 (83)	18 (82)	3 (75)	21 (81)	108 (83)

Abbreviations: TB= pulmonary tuberculosis, SD=standard deviation

5.3.1 Utility of Individual anti-*M.tb* Antibodies in the Diagnosis of TB Disease

Titres of IgA antibodies against six antigens (Apa, “Kit 1”, “Kit 2”, NarL, Rv3019c and PstS1) and titres of IgM antibodies against LAM (Table 2.3) were measured in a blinded manner, according to the instructions of the kit manufacturer, in plasma samples from 156 study participants. When the baseline levels of the antibodies detected in the TB patients (n=26) were compared to the levels obtained in the 130 individuals with ORD with the Mann-Whitney U test, IgA antibodies against four *M.tb* antigens (Rv3019c, “Kit 1”,”Kit 2” and NarL) differentiated significantly between active TB patients and individuals with ORD (Table 5.2, Figure 5.1). When the diagnostic accuracies of individual antibodies were investigated by ROC curve analysis, anti-NarL IgA was the only individual antibody that showed promise, with an area under curve (AUC) of 0.74 (95% CI, 0.64-0.83) and sensitivity and specificity of 92% (95% CI, 75-99%) and 52% (95% CI, 34-60%) respectively. anti-Rv3019c IgA and anti-“Kit 2” IgA performed with AUCs ≥ 0.66 (Table 5.2, Figure 5.1). When data was stratified according to HIV infection status the levels of anti-Psts1 IgA became significant between the two groups with an AUC of 0.63.

Table 5.2: Median optical density (OD $_{450}$) values (and inter-quartile ranges in parenthesis) and diagnostic accuracies of individual antibodies against *M.tuberculosis* antigens in plasma samples to distinguish between TB disease (n=26) and individuals with ORD (n=130).

M.tuberculosis antigens	Ig class	TB	ORD	p-value	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Cut-off Value
Rv3019c (TB10.3)	IgA	0.054 (0.03-0.07)	0.35 (0.02-0.05)	0.0029	0.69 (0.57-0.81)	54 (33-73)	82 (74-88)	> 0.0495
PstS1	IgA	0.044 (0.03-0.07)	0.034 (0.02-0.05)	0.07	0.61 (0.49-0.73)	77 (56-91)	48 (39-57)	> 0.0325
(Kit 1)	IgA	0.056 (0.04-0.08)	0.042 (0.03-0.06)	0.028	0.64 (0.52-0.76)	73 (52-88)	60 (51-68)	> 0.0465
(Kit 2)	IgA	0.058 (0.038-0.078)	0.041 (0.029-0.057)	0.011	0.66 (0.55-0.77)	54 (33-73)	76 (68-83)	> 0.0575
Apa	IgA	0.024 (0-0.037)	0.025 (0.006-0.044)	0.42	0.55 (0.43-0.67)	92 (75-99)	26 (18-35)	< 0.0410
NarL	IgA	0.066 (0.05-0.09)	0.040 (0.03-0.06)	0.0001	0.74 (0.64-0.83)	92 (75-99)	51 (34-60)	> 0.0405
LAM	IgM	0.073 (0.04-0.1)	0.071 (0.05-0.1)	0.98	0.5 (0.37-0.62)	73 (52-88)	35 (26-43)	< 0.0865

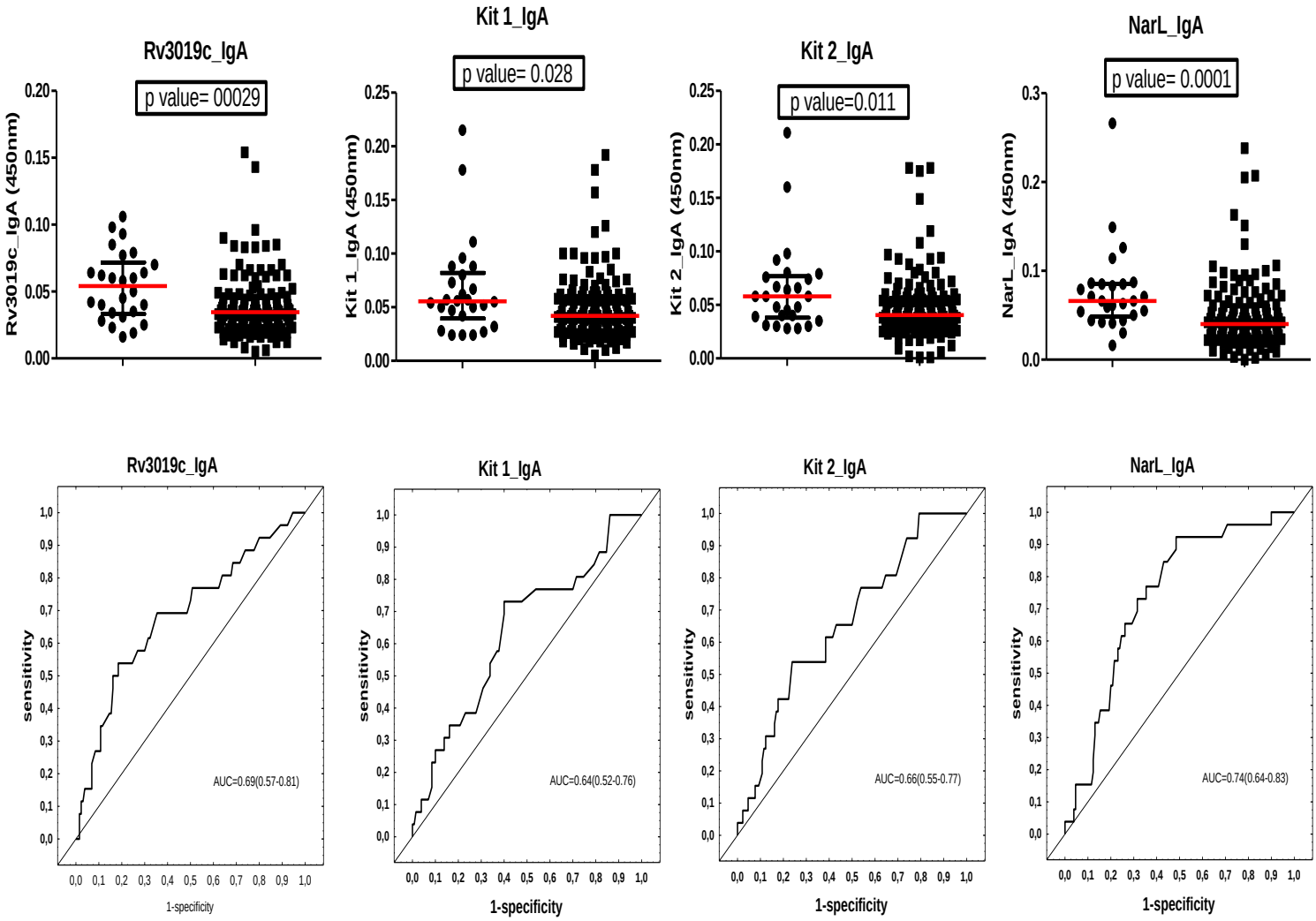

Figure 5.1: Scatter plots showing the median optical density (450nm) for anti-Rv3019c IgA, anti-"Kit 1" IgA, anti-"Kit 2" IgA and anti-NarL IgA in plasma samples from TB patients (n=26) and individuals with ORD (n=130) and receiver operator characteristics curves showing the accuracies of these antigens in the diagnosis of TB disease. Error bars in the scatter dot plots represent the median with interquartile range.

5.3.2 Utility of Multi-Antibody Models in the Diagnosis of TB Disease

When data obtained from all study participants were fitted into General Discriminant Analysis (GDA) models, regardless of HIV status, all seven antibodies investigated (anti-Rv3019c IgA+anti-PstS1 IgA+ anti-"Kit 1" IgA+ anti-"Kit 2" IgA+ anti-Apa IgA+ anti-NarL IgA+ anti-LAM IgM), when used in combination, discriminated between TB patients and individuals with ORD with AUC of 0.8 (95% CI, 0.72-0.88) (Figure 5.2).This resulted to a sensitivity of 65.4% (95% CI 44.4-82%) and specificity of 76.9% (95% CI, 68.6-83.7%) in the resubstitution classification matrix, and a sensitivity of 58% (95% CI, 37.2-76%) and specificity of 78% (95% CI, 66.9-82.3%) after leave-one- out cross validation. The positive and negative predictive values of the seven-marker antibody signature were 58% (95% CI, 37.2-76.0 %) and 75.3% (95% CI, 66.9-82.3 %) respectively after leave-one-out cross validation.

When the GDA procedure was repeated after excluding the HIV infected individuals the seven-marker antibody combination diagnosed TB disease with an AUC of 0.79 (95% CI, 0.7-0.89) (Figure 5.2)., corresponding to a sensitivity of 55% (95% CI, 32.7-74.9%) and specificity of 80.2% (95% CI,71.1-87.1%) in the resubstitution classification matrix, and sensitivity of 50% (95% CI, 37.2-76%) and specificity of 76%(95% CI, 66.9-82.3%) after leave one out cross validation (Figure 5.2).

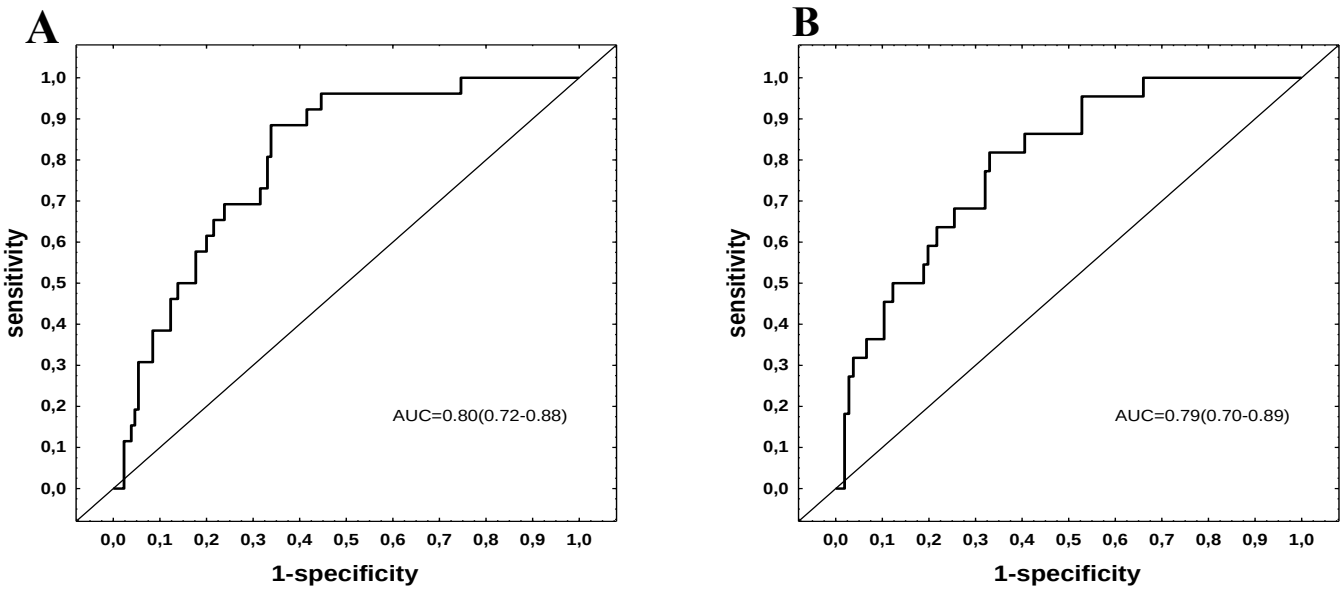

Figure 5.2: Accuracy of multi-marker models in the serodiagnosis of TB disease. Receiver operating curve characteristics (ROC) curve showing the accuracy of a seven-marker biosignature (anti-Apa IgA, anti- "Kit 1" IgA, anti-"Kit 2" IgA, anti- NarL IgA, anti-Rv3019c IgA, anti- PstS1 IgA and anti-LAM IgM) in all study participants, regardless of HIV infection (A), ROC curve showing the diagnostic accuracy of the seven-marker biosignature (anti-Apa IgA, anti-"Kit 1" IgA, anti-"Kit 2" IgA, anti- NarL IgA, anti-Rv3019c IgA, anti- PstS1 IgA and anti-LAM IgM) in HIV negative individuals (B).

5.3.3 Diagnostic Accuracy of Multi-Antibody Models when used in Combination with Cytokines

During the design of the present study, attempts were made at ensuring that there are common study participants between the study reported in Chapter 3 and the present study. This study design provided the opportunity for us to investigate the utility of combinations between inflammatory host markers (investigated in chapter 3) and the antibody responses investigated in the current chapter. A total of 46 study participants (20 culture confirmed TB patients and 26 individuals with ORD individuals) were common between chapter 3 and the present chapter. When the host inflammatory biomarker- and the antibody data obtained from these participants were fitted into GDA models, regardless of HIV status, a biosignature comprising of a combination between anti-"Kit 1" IgA and 5 host markers namely; neural cell

adhesion molecule (NCAM), vitronectin, complement factor H, ferritin and α-2 macroglobulin (A2M) diagnosed TB disease with a sensitivity of 95% (95% CI, 73-100%) and specificity of 88.5% (95% CI, 68.7-97%), both in the resubstitution classification matrix and after leave-one-out cross validation (Figure 5.3). The positive and negative predictive values of this biosignature was 86.4% (95% CI, 64-96.4%) and 95.8% (95% CI, 76.9-99.8%) respectively.

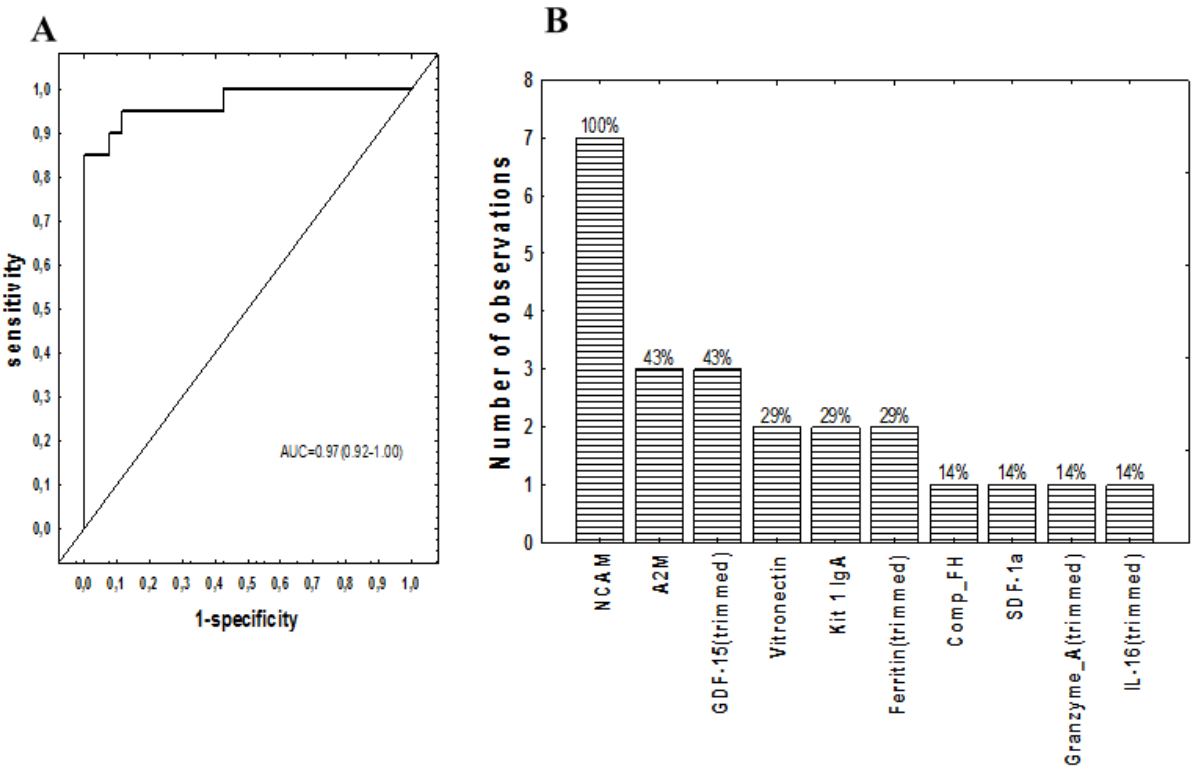

Figure 5.3: Accuracy of multi-marker models for host markers and antibody combinations for the diagnosis of TB disease. Receiver operating curve characteristics (ROC) curve showing diagnostic accuracy of the six-marker biosignature (anti-"Kit 1" IgA, NCAM, vitronectin, Complement factor H, ferritin and A2M in all participants, regardless of HIV infection (A), frequency of analytes in the top 7 general discriminant analysis (GDA) models that most accurately classified study participants as TB disease or ORD irrespective of HIV status (B).

5.3.4 Diagnostic Accuracy of Multi-Antibody Models when used in Combination with Symptoms

To investigate whether the use of antibody responses had the potential to contribute to the diagnosis of TB disease when used in combination with symptoms, antibody titres together with the symptoms (see table 5.1) that participants presented with at enrolment were fitted into GDA models. When night sweats and shortness of breath were combined with anti-“Kit 1” IgA, anti-“Kit 2” IgA, anti-Apa IgA and anti-NarL IgA responses, this antibody-symptom combination diagnosed TB disease with a sensitivity of 85% (95% CI, 64.3-95%) and specificity of 72% (95% CI, 62.8-78.9%) in the resubstitution classification matrix, and sensitivity and specificity of 85% (95%CI, 64.3-95%) and 71% (95% CI, 62-78.2%) after leave-one-out cross validation respectively. The positive and negative predictive values of this biosignature were 84. % (95% CI, 64.2-94.9%) and 70.8% (95% CI, 62.0-78.2%) respectively after leave-one out-cross validation (Figure 5.4). When the GDA procedure was repeated after excluding the HIV infected individuals the most optimal biosignature was a combination between anti-Apa IgA, NarL IgA, night sweats, malaise and shortness of breath, which discriminated between the TB disease and ORD group with an AUC of 0.81 (95% CI, 0.7-0.92) (Figure 5.4), corresponding to a sensitivity of 86.4% (95% CI, 64-96.4%) and specificity of 74.5% (95% CI, 65-96.4%) in the resubstitution classification matrix, and sensitivity and specificity of 82% (95% CI, 59-94%) and 73% (95% CI, 63-80.1%) respectively after leave-one-out cross validation. Anti-Apa IgA was the most frequent antibody and night sweats the most frequent symptom in GDA biosignatures, appearing in 54% and 46% of the biosignatures generated for diagnosing TB regardless of HIV infection status respectively (Figure 5.4).

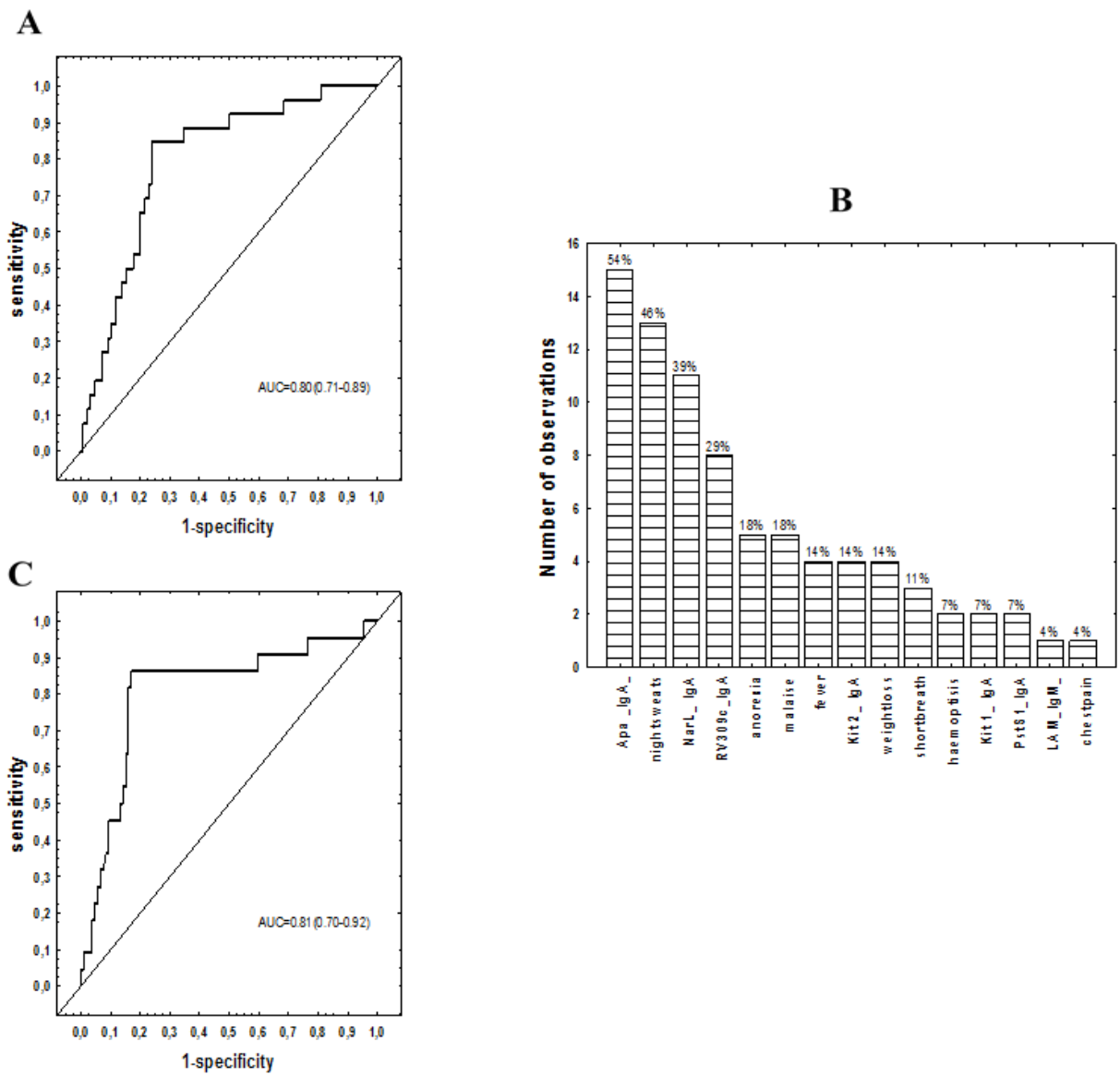

Figure 5.4: Accuracy of multi-marker models for antibody and symptoms combinations in the diagnosis of TB disease. Receiver operating curve characteristics (ROC) curve showing diagnostic accuracy of the six-marker biosignature (anti-"Kit 1" IgA, anti-"Kit 2" IgA, anti-Apa IgA, anti-NarL IgA, night sweats and short breath) in all participants, regardless of HIV infection (A), frequency of anlaytes in the top 28 general discriminant analysis (GDA) models that most accurately classified study participants as TB disease or ORD irrespective of HIV status (B),ROC curve showing the diagnostic accuracy of a five marker biosignature (anti-Apa IgA,anti- NarL IgA, night sweats, malaise and short breath) in HIV negative individuals (C).

5.3.5 Changes in Antibody Levels during the Course of TB Treatment

To investigate whether the levels of the antibodies investigated in the present study could potentially be used to monitor TB treatment response, we evaluated the antibody levels against five of the *M.tb* antigens (Rv3019c, PstS1, Apa, NarL and LAM) in plasma samples that were collected from TB patients at baseline, months 2 and 6, following the start of TB treatment. Samples collected at month 2 following the initiation of TB treatment were available from 25(96%) of the 26 TB patients, and from 21 (81%) of the patients after treatment completion (month 6). None of the antibodies showed significant changes in the course of treatment, when the OD values read at 450nm were considered. When a reference filter (650nm) was employed with the 450nm measurements, the significant changes were observed in the titres of four of the antibodies (Figure 5.5). The titres of anti- Rv3019c IgA increase significantly from baseline to month 2, whereas a decrease was observed towards the end of treatment. Anti-Psts1 IgA and anti-Apa IgA levels significantly decreased from month 2 to month 6, whereas anti-NarL IgA levels significantly decreased throughout the course of treatment (Figure 5.5).

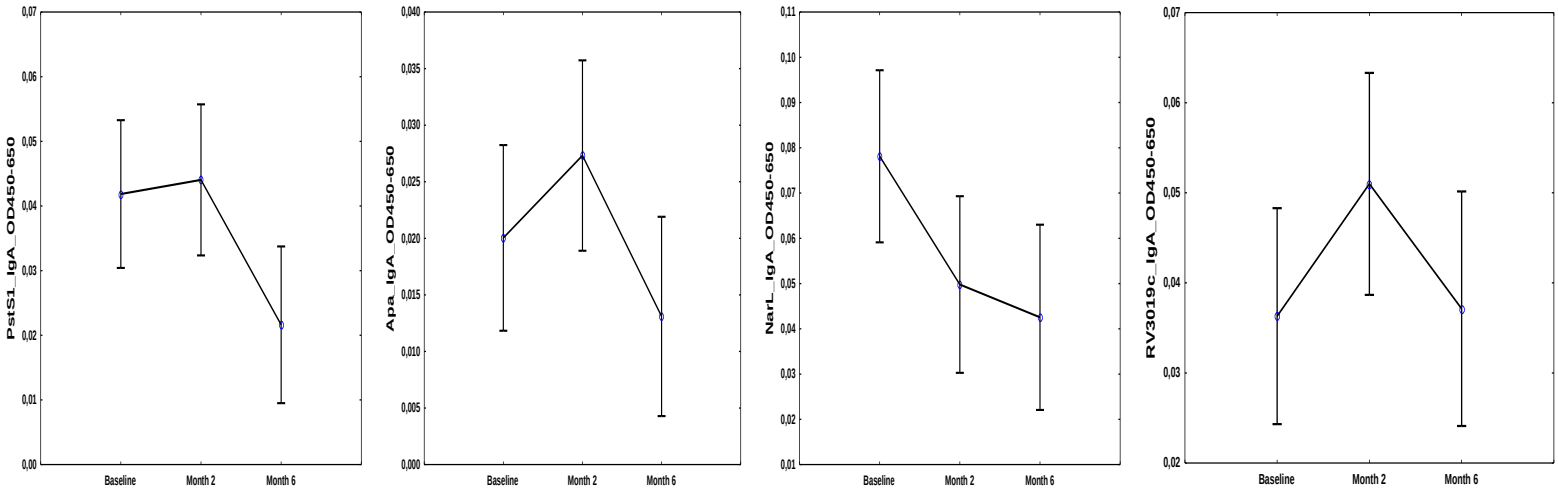

Figure 5.5: Baseline, month 2 and month 6 (after treatment) mean optical density (450nm-650nm) values of antibodies in plasma samples from TB patients. Error bars indicate the Least Squared means with 95% Confidence Intervals

5.4 Discussion

Serological diagnostic methods for TB disease have extensively been studied with variable success [79]. Although the World Health Organisation (WHO) advised against the use of commercial serological tests for the diagnosis of TB due their suboptimal performance in the negative policy document [70] it encouraged further investigations on strategies to improve the accuracies of these tests. Previous studies demonstrated a role for antibodies during protection against intracellular pathogens via various mechanisms [156, 157]. Antibodies could possibly mediate protection by preventing pathogens from entering cells or by enhancing the production of pro-inflammatory cytokines [158, 159]. In the case of TB de Valiere et al demonstrated that antibodies enhances cellular immune responses in order to protect the host [160]. In the present study, we evaluated the serodiagnostic potentials of antibodies against seven *M.tb* specific antigens (Apa, “Kit 1”, “Kit 2”, NarL, Rv3019c, PstS1 and LAM) for the immunological diagnosis of TB disease and monitoring of TB treatment response. IgA antibody responses against NarL, Rv3019c, “Kit 1”and“Kit 2” were significantly different between TB patients and individuals with ORD, with the most promising single antibody being anti-NarL IgA. We also demonstrated that the diagnostic performance of antibodies can be improved by using a combination of antigens as well as by combining antibody measurements with cytokine inflammatory biomarker measurements and clinical symptoms.

NarL has been described as a putative nitrate response regulator and is part of the membrane fraction of *M.tb* [161, 162]. IgA antibody responses against NarL in serum samples have previously been investigated for the diagnosis of active TB disease, and discriminated between active TB patients and healthy controls with 78.6% sensitivity and 100% specificity [163]. Our findings that IgA antibodies against NarL may be useful in the diagnosis of TB disease are therefore in keeping with these previous observations. When the TB-specific antibodies were used in combination with initial clinical symptoms that patients presented with at enrolment, the most optimal biosignature was a combination between anti-“Kit 1” IgA, anti-“Kit 2” IgA, anti-Apa IgA, anti-NarL IgA, night sweats and shortness of breath, which diagnosed TB disease with sensitivity and specificity of 85% and 72% respectively and the same AUC (0.80), was obtained as when multiple antibodies were used in combination.

Apa is an actively secreted antigen of *M.tb* and IgA responses against this antigen have previously been investigated. Apa diagnosed TB disease in serum samples with promising accuracy [78]. Furthermore, the antigen has been shown to be useful in the diagnosis of TB at the early stages of disease, and may also be useful in individuals co-infected with HIV [164]. Although Apa alone did not perform well in the current study, it was the most frequently appearing antigen in the multi-antigen biosignatures that were generated. This antigen was also included in an antigenic cocktail (Apa, CFP-10, ESAT-6, PstS-1 and Ag85) that was generated in a Cuban population for the diagnosis of active TB disease, and diagnosed TB with a sensitivity of 87.1% and specificity of 97.1% [165], thereby indicating the potential of this antigen in combination with other *M.tb* specific antigens as useful diagnostic tool for active TB.

With the aim of further improving the sensitivity and specificity for the diagnosis of TB disease, we also investigated an approach in which inflammatory host biomarker responses were combined with antibodies against these TB-specific antibodies. Several studies have demonstrated the potential usefulness of cytokines in the diagnosis of TB disease [82, 98, 100]. However, it is well-established that these markers are not particularly disease specific, as their levels are raised in multiple infections and non-infectious inflammatory conditions. A biosignature comprising of a combination between anti-"Kit 1" IgA and five cytokines (NCAM, vitronectin, Complement factor H, ferritin and A2M) diagnosed TB disease in the present study, with a sensitivity of 95% and specificity of 88.5%, and with a negative predictive value of 95.8%. NCAM has been found to be important in cell-cell or cell-matrix interactions [129] and its role in lung tumor progression has been described [130]. The potential of NCAM as a diagnostic marker for TB disease has recently been identified in a study conducted in our laboratory [147] (chapter 3 of this thesis) where NCAM was found to be to be the most frequently occurring biomarker in biosignatures for the diagnosis of TB disease. Two of the host markers included in the current biosignature (ferritin and A2M) are acute phase proteins that have previously been shown to have potential as biomarkers for the diagnosis of TB disease [111, 166]. Complement factor H is a soluble complement regulator important for controlling the alternative pathway [167] and has been included in a seven-marker biosignature for the diagnosis of TB disease in a serum- based study previously conducted in

our laboratory [82]. Taking the potential shown by these biomarkers in these previous studies as diagnostic candidates for TB disease into consideration, the findings from the present study indicate that the combination of host inflammatory protein biomarkers and TB-specific antibodies may enhance the diagnostic accuracy for TB disease. These observations require validation in future much larger studies.

When investigated as potential candidates for monitoring of the response to TB treatment, antibodies against four *M.tb* specific antigens (Psts1, Apa, NarL and Rv3019c) showed potential, with IgA antibody responses against the proteins changing significantly during the course of TB treatment. To the best of our knowledge these antigens have not previously been investigated for as potential markers for monitoring of TB treatment response. Therefore further investigations are required in order to validate their usefulness as indicators of the response to TB treatment. As with other new biomarkers identified in the course of the work presented in this thesis, these TB-specific antibody responses should also be investigated for their potential as predictors of month 2 culture status and in differentiating between different treatment outcomes including cure and particularly unfavourable outcomes including relapse and patients at increased chances of treatment failure. In addition for monitoring treatment response it might also be useful to look at antibody profiles together with cytokine profiles for evaluating an individual patient's progress throughout treatment.

Although the sample size investigated in the current study is considerably larger than sample sizes in most similarly published studies, our sample size of 152 may be seen as a limitation. Contrary to other published studies that were pure case-control studies, the current study made use of samples that were collected from individuals that presented at the primary health care centre with signs requiring investigation for TB, prior to the establishment of a clinical diagnosis. Our findings may therefore be very relevant but will require validation in larger studies, including studies conducted in other geographical regions. Incorporation of biosignatures comprising of anti-*M.tb* specific antibodies and host biomarkers into point-of-care screening test platforms may be very useful in the control of TB disease. However, this can only be done after future larger validation studies. Such future studies should also include

more HIV infected individuals and patients presenting with symptoms that are often confused with TB including sarcoidosis and pneumonia as these patient types will be useful in estimating the specificity of the biosignatures.

In conclusion we have identified biosignatures of anti-*M.tb* antibodies and combinations including antibodies and host inflammatory biomarkers which might be useful in the diagnosis of TB disease. The biosignatures identified in our study require further validation in larger multi-site prospective studies.

Chapter 6

Concluding Remarks

6.1 Overview

As highlighted in previous chapters, there is an urgent need for new tools for the rapid diagnosis of TB disease and monitoring of treatment response, especially in resource-constrained settings. As diagnostic tests based on *ex vivo* inflammatory host biomarkers have the potential to be easily translated into diagnostic tools which are simple, easy to use and which may be easily converted into point-of-care (POC) diagnostic tools which would be suitable in resource-limited settings, the primary focus of these thesis was in investigating such biomarkers in plasma and saliva samples from individuals with presumed TB disease. Saliva is a readily available sample, which can be collected easily from all patients, including children, whereas the utility of plasma based biomarkers could be investigated in finger-prick blood, which will also enable easier utility of such a test in all patients.

6.2 Summary of Main Findings

In Chapter 3, the concentrations of 74 host biomarkers were investigated in plasma samples, as potential markers for the diagnosis of TB disease and monitoring of the response to TB treatment. Eighteen host markers showed potential as diagnostic candidates for TB disease as ascertained by area under the ROC curve (AUC); with the most promising being NCAM, CRP, SAP, IP-10, ferritin, TPA, I-309, and MIG, which diagnosed tuberculosis disease individually, AUC ≥0.80. However, the main finding of the study was that a six-marker plasma protein biosignature comprising of NCAM, serum amyloid P, IL-1β, soluble CD40 ligand, IL-13 and apolipoprotein A-1 diagnosed TB disease with a sensitivity of 100% (95% CI, 86.3-100%) and specificity of 89.3% (95% CI, 67.6-97.3%), irrespective of HIV status. In the absence of HIV infection, two different six-marker biosignatures comprising of NCAM, alpha-2-macroglobulin, IL-22, ferritin, myoglobulin, IL-12(p40), and NCAM, alpha-2-macroglobulin, IL-22, ferritin, TNF-β and MIP-4, diagnosed TB disease with both sensitivity and specificity of

100% (AUC = 1.0, 95% CI, 1.0-1.0). Furthermore, the concentrations of 11 of these host markers changed significantly during the course of treatment, thereby demonstrating their potential as tools to monitor TB treatment. In Chapter 4, we evaluated the concentrations of 69 host markers as salivary biomarkers for the diagnosis of TB disease or monitoring of the response to treatment. Of the 69 host markers that were investigated, the concentrations of 23 were not detectable or just barely detectable, whereas eight were significantly different in saliva samples from TB patients and individuals with ORD. Although two markers (IL-16 and IL-23) showed promise individually as diagnostic candidates for TB disease as determined by AUC ≥0.70, the main finding of the study was a five-marker biosignature comprising of IL-1β, IL-23, ECM-1, HCC1 and fibrinogen which diagnosed TB disease with a sensitivity of 88.9% (95% CI,76.7-99.9%) and specificity of 89.7% (95% CI, 60.4-96.6%) after leave-one-out cross validation, regardless of HIV infection status, whereas eight-marker biosignatures performed with a sensitivity of 100% (95% CI, 83.2-100%) and specificity of 95% (95% CI, 68.1-99.9%) in the absence of HIV infection. Furthermore, the concentrations of 8 of the markers changed during treatment, indicating that salivary biomarkers may be useful in monitoring of TB treatment response. In Chapter 5, the diagnostic potential of antibodies against seven *M.tb* specific antigens namely; Apa, NarL, Rv3019c, PstS1, LAM and two other antigens whose identities have not yet been disclosed due to intellectual property concerns ("Kit 1" and "Kit 2"), were investigated as tools for the diagnosis of TB disease and monitoring of the response to treatment. IgA antibody responses against four antigens (NarL, Rv3019c, "Kit 1" and "Kit 2") were significantly different between TB patients and individuals with ORD, with anti-NarL antibodies showing the most promise individually as diagnostic candidates, with an AUC of 0.74, corresponding to a sensitivity of 92% (95% CI, 75-99%) and specificity of 52% (95% CI, 34-60%). When antibodies against all seven proteins investigated were used in combination, the seven-antibody biosignature ascertained TB disease with an AUC of 0.8. When antibodies against the antigens were used in combination with clinical symptoms, anti-"Kit 1" IgA, anti-"Kit 2" IgA, anti-Apa IgA, anti-NarL IgA in combination with night sweats and shortness of breath diagnosed TB disease with sensitivity and specificity of 85% (95%CI, 64.3-95%) and 71% (95% CI, 62-78.2% respectively after leave-one-out cross validation. When antibody responses were used in combination with host inflammatory biomarkers ("cytokines"), a six-marker biosignature comprising of a combination between anti-"Kit 1" IgA and five host markers (NCAM, vitronectin, complement factor H, ferritin and A2M diagnosed TB disease

with a sensitivity of 95% (95% CI, 73-100%) and specificity of 88.5% (95% CI, 68.7-97%) after leave-one-out cross validation. As observed with plasma and salivary host inflammatory biomarkers, antibodies against four of the *M.tb* specific antigens (Psts1, Apa, NarL and Rv3019c) also showed potential as candidate markers for TB treatment response.

6.3 Significance of Findings from this Thesis:

In this thesis, host inflammatory biomarkers detected in plasma and saliva as well as plasma antibodies against *M.tb* antigens were identified as diagnostic candidates for TB disease. Although individual host markers and antibodies showed promise, biomarkers identified in the different chapters of the thesis always performed better when used in combination. This therefore stresses the importance of any future TB diagnostic tests that could be developed from these biomarkers, to be based on combinations of analytes, rather than individual host markers and antibodies. As observed in chapter five, combinations between different classes of biomarkers (antibodies against *M.tb* antigens, used in combination with cytokines or symptoms) shows promise as a diagnostic approach. The fact that the biosignatures identified in the present thesis were detectable in relatively non-invasive samples (plasma and saliva), is important, as such samples can be relatively easily collected from all patient types, even in remote settings. It will be ultimately most beneficial if these markers are incorporated into POC tests, which are suitable for use at the primary health care level, especially in high TB burdened and resource-constrained settings. Such tests should ideally be cost-effective lateral flow point-of-care tests that are capable of delivering results in minutes, on the same day. The earlier and faster diagnosis of TB disease implies that patients could start treatment earlier (ideally on the same day that they presented at the health care centre) and this will prevent further transmission of *M.tb* to their contacts. This thesis also demonstrated the potential of host markers and antibody responses against *M.tb* specific antigens as candidates to monitor treatment response. The use of these markers would therefore aid in knowing whether a patient is responding to treatment or whether the course of treatment should be altered. If such markers and antibodies are measured earlier, it will be informative and therefore more beneficial to TB control programs than the current

situation where a patient's response to treatment is only assessed at month 2, and mostly using the poorly sensitive sputum smear microscopy test. These markers, if validated in future studies, will be also beneficial to TB drug manufacturers as it will be easier to assess if a new TB drug is working or not, thereby leading to shortening of the duration of clinical trials. However, the findings from this thesis are still preliminary as more validation studies still needs to be done.

Overall, the diagnostic biosignatures identified in the present thesis performed with high negative predictive values. This means that these biosignatures, if incorporated into POC tests, would be useful as rule out tests for TB disease. Such a POC test would be able to identify patients that require confirmatory testing such as with the well-established tests (culture and GeneXpert), and may help in limiting the number of expensive tests usually performed in individuals suspected of having TB disease, as only about 30% of these individuals are finally diagnosed with TB. This approach will therefore lead to major cost savings. Such a POC test that is suitable for use at community level clinics is an urgent need as highlighted in the WHO target product profiles for new tests document that was published in 2014. The use of these ex vivo samples implies that such tests will be useful in diagnosing TB disease in patients having difficulty in providing good quality sputum, as well as diagnosing extrapulmonary TB, without the need for invasive techniques. However, further validation studies are required to refine the diagnostic biosignatures.

As cytokines are produced by various immune cells, which influence the outcome of mycobacterial infection, the findings from this thesis have also helped in a better understanding of the pathogenicity of *M.tb,* specifically the host-pathogen interactions. Such interactions may provide insights into mechanisms of virulence, which could lead to innovative approaches for immunological intervention in TB disease. However, the investigation of the mechanisms of the disease, or the mechanisms influencing host inflammatory biomarker or antibody production were not the aim of the current thesis.

6.4 Future Investigations

The main common limitation amongst all studies conducted as part of this thesis was the limited sample size. Although the sample sizes were not smaller than the sample sizes that are used in most published studies, it will be important that future studies are done in larger sample sizes. Furthermore, it is important that such studies include study participants recruited from different geographical regions. This will ensure that the refined and validated biosignatures and any tests developed based on the biosignatures are of global relevance. It will also be important that more HIV infected individuals are included into such future studies. As discussed in previous chapters, it will be important if HIV infected participants included in such future studies are staged with CD4 cell counts and viral loads, so as to investigate the influence of severe HIV infection on the accuracy of the host biosignatures. Future investigations in patients with other comorbidities such as diabetes would also be of significance. Furthermore, the TB patients investigated in this thesis were all adults, with confirmed pulmonary TB. Therefore it cannot be ascertained from the findings of the current thesis whether these host biosignatures would be useful in children with paediatric TB, and those with extrapulmonary disease. There is therefore a need for investigation of the diagnostic potential of the identified biosignatures in children and in patients with extrapulmonary TB disease. Furthermore, as previously discussed in the thesis, it will also be important if patients presenting with confirmed diseases that are similar to TB, including non-TB pneumonias are included in future studies. This is because the ORD groups investigated in this thesis lacked firmly established alternate diagnoses, even though they all presented at the clinic with symptoms requiring investigation for TB. Such a group of study participants would be important in order to ascertain the specificity of the host biosignatures for the diagnosis of TB. Further larger studies would also be required to investigate the potential of host markers as tools to monitor the response to TB treatment, and should also include samples that are recruited at earlier time points. Additional investigations should also look at the abilities of the host markers to predict month 2 culture status as well as final treatment outcome (clinical cure, relapse or treatment failure). It will be important that markers identified in plasma are also investigated in finger-prick blood, as this approach is planned to be investigated in a study that is currently ongoing in the Stellenbosch University immunology research laboratory as well as laboratories in other African and European countries

(www.screen-tb.eu), the markers identified in the current thesis could form part of the panels planned to be investigated in such larger studies, with the ultimate aim being the development of a POC test that makes use of finger-prick blood.

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